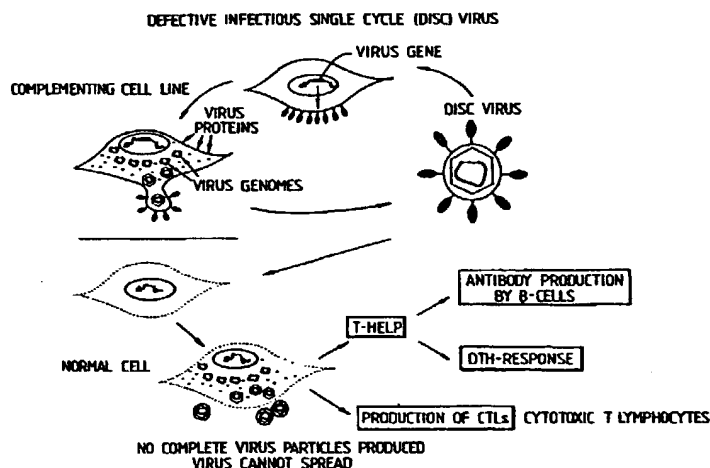




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(54) Title: DEFECTIVE MUTANT NON-RETROVIRAL VIRUS (E.G. HSV) AS VACCINE



(57) Abstract

The application provides a pharmaceutical which comprises a mutant non-retroviral virus (particularly HSV-1 and/or HSV-2) whose genome is defective in respect of a gene essential for the production of infectious virus. The virus can infect normal cells and undergo replication and expression of viral antigen genes in those cells but cannot produce normal infectious virus. The pharmaceutical is for prophylactic or therapeutic use in generating an immune response in a subject infected therewith. Where the non-retroviral virus is a herpes simplex virus e.g. HSV-1 or HSV-2, the defect can be in the glycoprotein gH gene. Vaccines and therapeutic pharmaceuticals are provided especially for epithelial, oral, vaginal and nasal administration. Also provided is use of a mutant based on HSV-1 for the preparation of a pharmaceutical for prophylactic or therapeutic use in generating an immune response in a subject against type-2 HSV infection.

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DEFECTIVE MUTANT NON-RETROVIRAL VIRUS (E.G. HSV) AS VACCINE.

The present invention relates to viral vaccines.

Viral vaccines are traditionally of two sorts. The first sort are 'killed' vaccines, which are virus preparations which have been killed by treatment with a suitable chemical such as beta-propiolactone. The second type are live 'attenuated' vaccines, which are viruses which have been rendered less pathogenic to the host, either by specific genetic manipulation of the virus genome, or, more usually, by passage in some type of tissue culture system. These two types of vaccine each have their own disadvantages. Since killed vaccines do not replicate in the host, they are usually administered by injection, and hence may generate an inappropriate kind of immune response. For example the Salk vaccine, a killed preparation of poliovirus, produces an immunoglobulin (Ig) G antibody response, but does not stimulate the production of IgA in the gut, the natural site of primary infection. Hence this vaccine, though it can protect the individual from the neurological complications of poliomyelitis, does not block primary infection, and so does not confer "herd immunity". In addition, killed viruses, do not enter and replicate inside host cells. Hence any beneficial immunological response to non-structural proteins produced during replication is not available. They also often fail to stimulate the production of cytotoxic T cells directed against virus antigens. "Dead" antigens can be picked up by antigen presenting cells and presented to T cells. However, the presentation occurs via MHC Class II molecules

and leads to stimulation of T helper cells. In turn, the T helper cells help B cells to produce specific antibody against the antigen. In order to stimulate the production of cytotoxic T cells, virus antigens must be processed through a particular pathway inside the infected cell, and presented as broken-up peptide fragments on MHC Class I molecules. This degradation pathway is thought to work most effectively for proteins that are synthesised inside the infected cell, and hence only virus that enters host cells and expresses immunogenic viral protein is capable of generating virus-specific cytotoxic T cells. Therefore, killed vaccines are poor inducers of cytotoxic T cells against virus infection. From this point of view, live attenuated vaccines are more satisfactory.

To date, live attenuated viruses have been made by deleting an inessential gene or partly damaging one or more essential genes (in which case, the damage is such that the genes are still functional, but do not operate so effectively). However, live attenuated viruses often retain residual pathogenicity which can have a deleterious effect on the host. In addition, unless the attenuation is caused by a specific deletion, there remains the possibility of reversion to a more virulent form. Nevertheless, the fact that some viral protein production occurs in the host means that they are often more effective than killed vaccines which cannot produce such viral protein.

Live attenuated viruses, as well as being used as vaccines in their own right, can also be used as 'vaccine

vectors' for other genes, in other words carriers of genes from a second virus (or other pathogen) against which protection is required. Typically, members of the pox virus family eg. vaccinia virus, are used as vaccine
5 vectors. When a virus, is used as a vaccine vector, it is important that it causes no pathogenic effects. In other words it may need to be attenuated in the same way that a simple virus vaccine is attenuated. The same disadvantages as those described above, therefore apply in this case.

10 It has been found possible to delete an essential gene from a viral genome whilst also providing a so-called 'complementing' cell which provides the virus with the product of the deleted gene. This has been achieved for certain viruses, for example adenoviruses, herpesviruses
15 and retroviruses. For adenoviruses, a human cell line was transformed with fragments of adenovirus type 5 DNA (Graham, Smiley, Russell & Nairn, J. Gen. Virol., 36, 59-72, 1977). The cell line expressed certain viral genes, and it was found that it could support the growth of virus
20 mutants which had those genes deleted or inactivated (Harrison, Graham & Williams, Virology 77, 319-329, 1977). Although the virus grew well on this cell line (the 'complementing cell line') and produced standard viral particles, it could not grow at all on normal human cells.
25 Cells expressing the T-antigen-encoding region of the SV40 virus genome (a papovavirus) have also been shown capable of supporting the replication of viruses specifically deleted in this region (Gluzman, Cell, 23, 182-195, 1981).

For herpes simplex virus, cell lines expressing the gB glycoprotein (Cai et al, J. Virol. 62, 714-721, 1987) the gD glycoprotein (Ligas and Johnson, J. Virol. 62, 1486, 1988) and the Immediate Early protein ICP4 (Deluca et al., J. Virol., 56, 558, 1985) have been produced, and these have been shown capable of supporting the replication of viruses with specifically inactivated copies of the corresponding genes.

W092/05263 published on 2 April 1992 provides a mutant non-retroviral virus whose genome is defective in respect of a gene essential for the production of infectious virus, such that the virus can infect normal cells and undergo replication and expression of viral antigen genes in those cells but cannot produce normal infectious virus.

Mutant non-retroviral viruses in accordance with the teaching of W092/05263 provide a unique way of combining the efficacy and safety of a killed vaccine with the extra immunological response induced by the in vivo production of viral protein by the attenuated vaccine. In preferred embodiments, the invention of W092/05263 comprises two features. Firstly, a selected gene is inactivated within the virus genome, usually by creating a specific deletion. This gene will be involved in the production of infectious virus, but preferably not preventing replication of the viral genome. Thus the infected cell can produce more viral protein from the replicated genetic material, and in some cases new virus particles may be produced, but these would not be infectious. This means that the viral

infection cannot spread from the site of inoculation.

A second feature of the invention of W092/05263 is a cell which provides the virus with the product of the deleted gene, thus making it possible to grow the virus in tissue culture. Hence, although the virus lacks a gene encoding an essential protein, if it is grown in the appropriate host cell, it will multiply and produce complete virus particles which are to outward appearances indistinguishable from the original virus. This mutant virus preparation is inactive in the sense that it has a defective genome and cannot produce infectious virus in a normal host, and so may be administered safely in the quantity required to generate directly a humoral response in the host. Thus, the mutant virus need not be infectious for the cells of the host to be protected and merely operates in much the same way as a conventional killed or attenuated virus vaccine. However, preferably the immunising virus is itself still infectious, in the sense that it can bind to a cell, enter it, and initiate the viral replication cycle and is therefore capable of initiating an infection within a host cell of the species to be protected, and producing therein some virus antigen. There is thus the additional opportunity to stimulate the cellular arm of the host immune system.

In particular, it is to be mentioned that W092/05263 provided in vivo data which showed that intra-epithelial vaccination of mice via the ear with a mutant form (as described above) of HSV-1 gave better protection against

later challenge with wild-type HSV-1, than similar vaccination with killed HSV-1. A clear protective effect against the establishment of latent infection in the cervical ganglia was also shown for vaccination with the mutant HSV-1.

The applicants call the above described mutant viruses DISC viruses (standing for 'defective infectious single cycle) and the basic concept is illustrated in Fig. 1. The present application goes on from the work disclosed in W092/05263.

The present application makes the disclosures summarised below.

(1) In a study using the mouse ear model the results reported in W092/05263 were confirmed. Intra-epithelial vaccination of mice with DISC HSV-1 led to complete protection against replication of the challenge virus wild type (w.t.) HSV-1. Little effective protection was provided by equivalent doses of inactivated HSV-1. DISC HSV-1 also protected against the establishment of latent infection in the cervical ganglia.

(2) Also in the mouse ear model it is shown that no significant differences in antibody titres were observed between animals vaccinated with DISC HSV-1 and an equivalent amount of inactivated HSV-1.

(3) Also in the mouse ear model it is shown that at low vaccination doses, inactivated HSV-1 failed to establish a delayed-type hypersensitivity (DTH) response, whilst equivalent doses of DISC HSV-1 established a DTH

response. At high doses, both DISC HSV-1 and inactivated HSV-1 induced similar DTH responses.

(4) Also in a mouse study it was shown that in contrast to vaccination with inactivated HSV-1, vaccination with DISC HSV-1 induced HSV-1 specific cytotoxic T cell activity.

(5) The in vivo mouse ear model was used to study long term prophylactic effect of DISC HSV-1. Two vaccinations of DISC HSV-1 was found to provide better long term protection against challenge with w.t. HSV-1 than two vaccinations of inactivated DISC HSV-1

(6) The in vivo mouse ear model was used to investigate the prophylactic effect of DISC HSV-2 against HSV-2 infection. Intra-epithelial vaccination of mice with DISC HSV-2 provided better protection against replication of the challenge virus w.t. HSV-2 than inactivated DISC HSV-2.

(7) The in vivo guinea-pig vaginal model was used to study the prophylactic effect of DISC HSV-1 against HSV-2 infection. It was shown that intra-epithelial or intra-vaginal vaccination with DISC HSV-1 provided a high degree of protection against the primary symptoms of HSV-2 infection. Immunisation with DISC HSV-1 or inactivated virus retarded growth of challenge virus w.t. HSV-2 in the vagina. Further intra-vaginal vaccination with DISC HSV-1 lessened the number of recurrent HSV-2 lesions in a 100 day follow-up period. Intra-epithelial vaccination with DISC HSV-1 and inactivated virus also resulted in reduced

recurrent lesions, but compared to intra-vaginal vaccination with DISC HSV-1, the reduction was less.

(8) Oral and intranasal vaccination of guinea-pigs with DISC HSV-1 led to protection against acute disease symptoms following challenge with w.t. HSV-2. The intranasal route appeared to be more effective than the oral route.

The per vaginum vaccination route in comparison to oral or intra-nasal vaccination resulted in significantly lower levels of recovered virus following challenge.

(9) In guinea-pigs which had recovered fully from primary HSV-2 disease, the therapeutic administration of DISC HSV-1 either intra-vaginally or intra-epithelially resulted in an apparent reduction in the frequency of recurrent of disease symptoms compared with mock vaccinated animals.

(10) In guinea-pigs which had recovered fully from primary HSV-2 disease, intra-vaginal therapeutic administration of DISC HSV-2 was more effective in reducing the frequency of recurrence of disease symptoms than treatment with DISC HSV-1.

The present invention provides a pharmaceutical mutant which comprises a mutant non-retroviral virus whose genome is defective in respect of a gene essential for the production of infectious virus such that the virus can infect normal cells and undergo replication and expression of viral antigen genes in those cells but cannot produce normal infectious virus, for prophylactic or therapeutic

use in generating an immune response in a subject infected therewith.

The defect may allow the production and release from the cells of non-infectious viral particles.

5 The present invention provides a pharmaceutical which comprises a mutant non-retroviral virus whose genome is defective in respect of a gene essential for the production of infectious virus such that the virus can infect normal cells and replicate therein to give rise to the production
10 and release from the cells of non-infectious viral particles. The pharmaceutical may be a vaccine capable of protecting a patient immunised therewith against infection or the consequences of infection by a non-retroviral virus. The pharmaceutical may be a vaccine capable of protecting a
15 patient immunised therewith against infection or the consequences of infection by the corresponding wild-type virus.

 The pharmaceutical may be a therapeutic capable of treating a patient with an established non-retroviral virus
20 infection. The pharmaceutical may be a therapeutic capable of treating a patient with an infection established by the corresponding wild-type virus.

 The pharmaceutical may be sub-cutaneously, intra-muscularly, intra-dermally, epithelially-, (with or without
25 scarification), nasally-, vaginally-, or orally-administrable comprising excipients suitable for the selected administration route.

 The mutant may be from a double-stranded DNA virus.

The mutant may be from a herpes virus. The mutant may be from a herpes simplex virus (HSV).

The mutant may be a type-1 HSV or a type-2 HSV. The defect may be in the glycoprotein gH gene.

5 The present invention provides a type-2 HSV whose genome is defective in respect of a gene essential for the production of infectious HSV-2 such that the virus can infect normal cells and undergo replication and expression of viral antigens in those cells but cannot produce normal
10 infectious virus, for prophylactic or therapeutic use in generating an immune response in a subject infected with HSV eg HSV-2.

The mutant HSV-2 defect allows the production and release from the cells of non-infectious virus particles.

15 Also provided is a type-2 HSV whose genome is defective in respect of a gene essential for the production of infectious HSV-2 such that the virus can infect normal cells and replicate therein to give rise to the production and release from the cells of non-infectious viral
20 particles.

The mutant may be capable of protecting a patient immunised therewith against infection or the consequences of infection with HSV eg infection by the corresponding wild-type virus.

25 The mutant may be capable of treating a patient with an established HSV infection eg infection by the corresponding wild-type virus.

The defect may be in the glycoprotein gH gene.

The present invention also provides use of a mutant type-1 HSV whose genome is defective in respect of a gene essential for the production of HSV-1 such that the virus can infect normal cells and undergo replication and expression of viral antigen genes in those cells but cannot produce normal infectious virus, for preparation of a pharmaceutical for prophylactic or therapeutic use in generating an immune response in a subject against type-2 HSV infection.

10 The use may be in respect of pharmaceuticals for intra-epithelial (with or without scarification), intra-vaginal, intra-nasal or per-oral administration.

15 The present invention also provides an assembly comprising a pharmaceutical (for prophylaxis ie a vaccine or for therapy ie a therapeutic) as described above in a container preferably a pre-filled syringe or glass vial/ampoule with printed instructions on or accompanying the container concerning the administration of the pharmaceutical to a patient to prevent or treat conditions
20 caused by HSV infection. The printed instructions may concern the prevention or treatment of facial or genital lesions.

25 Vaccines containing the mutants as described can be prepared in accordance with methods well known in the art wherein the mutant is combined in admixture with a suitable vehicle. Suitable vehicles include, for example, saline solutions, or other additives recognised in the art for use in compositions applied to prevent viral infections. Such

vaccines will contain an effective amount of the mutant as hereby provided and a suitable amount of vehicle in order to prepare a vaccine useful for effective administration to the host.

5 Dosage rates can be determined according to known methods. For example, dosage rate may be determined by measuring the optimum amount of antibodies directed against a mutant resulting from administration of varying amounts of the mutant in vaccine preparations. Attention is
10 directed to New Trends and Developments in Vaccines, Editors: A. Voller and H. Friedman, University Park Press, Baltimore, 1978 for further background details on vaccine preparation.

 Therapeutics comprising a mutant as herein provided
15 can be formulated according to known methods to provide therapeutically useful compositions, whereby the mutant is combined in admixture with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulation are described in Remington's Pharmaceutical Science by E.
20 W. Martin. Such compositions will contain an effective amount of the mutant hereof together with a suitable amount of carrier vehicle in order to prepare therapeutically acceptable compositions suitable for effective administration to the host.

25 Typically vaccines are prepared as injectables, (traumatic or non-traumatic) either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be

prepared. Preparations may also be encapsulated in liposomes. The active immunogenic ingredients are often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, trehalose, or the like and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as other stabilisers and/or pH buffering agents, which enhance the stability and thus the effectiveness of the vaccine.

The vaccines may be administered parenterally, by injection, for example, subcutaneously, intraepithelially (with or without scarification). Additional formulations which are suitable for other modes of administration eg oral, vaginal and nasal formulations are also provided. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of trehalose mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. The compositions may take the form of solutions, suspensions, tablets, pills, capsules sustained release formulations or powders.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be prophylactically effective. The quantity to be administered will have been predetermined from preclinical and clinical (phase I) studies to provide the optimum immunological response.

The vaccine may be given in a single dose schedule, or preferably in a multiple dose schedule. A multiple dose schedule is one in which a primary course of vaccination may be with 1-3 separate doses, followed by other doses
5 given at subsequent time intervals required to maintain and or re-enforce the immune response, for example, at 1-4 months for a second dose, and if needed, a subsequent dose(s) after several months. The dosage regimen will also, have been determined from preclinical and clinical
10 studies as maintaining the optimum immunological response over time.

In order that the invention is more clearly understood, it will be further described by way of example only, and not by way of limitation, with reference to the
15 following figures.

Figure 1 illustrates the DISC virus concept.

Figure 2 shows clearance of wild-type HSV-1 (w.t. HSV-1) strain SC16 virus in the ears of mice vaccinated with either live DISC HSV-1 or inactivated (β -propiolactone
20 treated) w.t. HSV-1 (strain SC16). Groups of 4 mice were vaccinated at the doses indicated by scarification of the left ear pinna. Mice were challenged 14 days post-vaccination with 2×10^6 pfu w.t. HSV-1 strain SC16 in the right ear pinna and virus titres were measured 5 days post
25 challenge. Data are expressed as the geometric means and standard errors of the means.

Figure 3 shows measurement of titres of neutralising and ELISA antibody to w.t. HSV-1 in mice vaccinated with

either w.t. HSV-1 (strain SC16), live DISC HSV-1, killed DISC HSV-1 or PBS. Sera from mice were assayed in the presence of complement for neutralising antibodies to w.t. HSV-1 in a plaque reduction assay. Individual titres are expressed as the reciprocal dilution of sera required to neutralise 50% of the infectivity obtained in the absence of antibody.

Figure 4 shows delayed-type hypersensitivity (DTH) responses in mice vaccinated with either w.t. HSV-1 (strain SC16), live DISC HSV-1, killed DISC HSV-1 or PBS. Mice were vaccinated in the left ear pinna at the doses indicated 14 days prior to challenge with 10^6 pfu w.t. HSV-1 (strain SC16) in the opposite ear. Ear thickness was measured 24 and 48 hours post-challenge and is expressed as the difference between the challenged and vaccinated ear. Data are presented as the means of differences in ear thickness (in μm).

Figure 5 shows cytotoxic T cell (CTL) responses in mice vaccinated with either live DISC HSV-1, killed DISC HSV-1, MDK (a thymidine kinase negative HSV-1 strain) or PBS. Mice were immunised twice intraperitoneally three weeks apart and cell suspensions made from spleens 10 days after the second injection. Cells were stimulated in vitro for 4 days before being tested in a CTL assay using ^{51}Cr -labelled A20/2J as target cells. Data are presented as mean % ^{51}Cr release from quadruplicate samples at each point. Standard errors of the means are all $<10\%$.

Figure 6 shows clinical symptoms as assessed by

erythema score in guinea-pigs post challenge with $10^{5.2}$ pfu w.t. HSV-2 (strain MS) subsequent to vaccination with doses of 2×10^7 pfu DISC HSV-1 at a 3 week interval either by the intra-epithelial or the intra-vaginal route;

5 Figure 7 shows clinical symptoms as assessed by total lesion score in guinea-pigs post challenge with $10^{5.2}$ pfu w.t. HSV-2 (strain MS) subsequent to vaccination with doses of 2×10^7 pfu DISC HSV-1 at a 3 week interval either by the intra-epithelial or the intra-vaginal route.

10 Figure 8 shows post challenge virus w.t. HSV-2 (strain MS) replication in guinea-pigs post challenge with $10^{5.2}$ pfu w.t. HSV-2 (strain MS) subsequent to vaccination with doses of 2×10^7 pfu DISC HSV-1 at a 3 week interval either by the intra-epithelial or the intra-vaginal route.

15 Figures 9a and 9b show recurrent disease in guinea-pigs post challenge with $10^{5.2}$ pfu w.t. HSV-2 (strain MS) subsequent to vaccination with doses of 2×10^7 pfu DISC HSV-1 at a 3 week interval by the intra-epithelial or the intra-vaginal route. Fig. 9a shows recurrent disease as
20 the cumulative mean erythema index per animal. Fig. 9b shows recurrent disease as cumulative mean number of days with disease per animal.

Figure 10 shows mean lesion score per animal (guinea-pigs) with w.t. HSV-2 (strain MS) infection and which have
25 been vaccinated via the vaginal, oral or nasal routes with a mock virus preparation, DISC HSV-1 or inactivated DISC HSV-1.

Figure 11 shows mean erythema score per animal

(guinea-pigs) with w.t. HSV-2 (strain MS) infection and which have been vaccinated via the vaginal, oral or nasal routes with a mock virus preparation, DISC HSV-1 or inactivated DISC HSV-1.

5 Figure 12 shows the mean log titre of w.t. HSV-2 (strain MS) per animal (guinea-pigs) with w.t. HSV-2 (strain MS) infection and which have been vaccinated via the vaginal, oral or nasal routes with a mock virus preparation, DISC HSV-1 or inactivated DISC HSV-1.

10 Figure 13 shows recurrent disease following therapeutic vaccination. This is shown as mean cumulative number of days on which disease was observed (disease/days) in groups of guinea-pigs vaccinated with DISC HSV-1 either intra-epithelially or intra-vaginally or with a mock virus
15 preparation intra-vaginally after challenge with w.t. HSV-2 (strain MS). Disease was classified as either presence of one or more lesions or an erythema score of 1 or more. Animals were monitored from 4 weeks after initial challenge with w.t. HSV-2 (strain MS) (day 0) for 100 days. Animals
20 were vaccinated at Day 0, Day 24 and Day 44 with 2×10^7 pfu or equivalent dose as indicated.

 Figure 14 relates to the long-term protective effect in mice of vaccination with DISC HSV-1 against challenge with w.t. HSV-1 (strain SC16). The graph shows the mean
25 log titre of w.t. HSV-1 in the ears 5 days post challenge and 223 days post vaccination.

 Figure 15 relates to the long-term protective effect in mice of vaccination with DISC HSV-1 against challenge

with w.t. HSV-1 (strain SC16). The graph shows neutralising antibody titres days 15, 27, 90, 152 and 218 post vaccination as stated.

5 Figure 16 relates to the protective effect in mice of vaccination with DISC HSV-2 against challenge with w.t. HSV-2 (strain HG52) for vaccinations with live DISC HSV-2, killed DISC HSV-2 and w.t. HSV-2 (strain HG52) at varying doses, the graph shows mean log titre of w.t. HSV-2 in the ear post challenge.

10 Figure 17 illustrates the construction of a single plasmid containing the complete HSV-2 gH gene.

Figure 18 shows the sequence of HSV-2 strain 25766 in the region of the gH gene including a translation of the gH gene in single letter amino acid code.

15 Figure 19 shows a comparison of the DNA sequence of HSV-1 and HSV-2 strain 25766 in the region of the gH gene.

Figure 20 shows a comparison of the deduced amino acid sequences of the HSV-1 strain 17 and HSV-2 strain 25766 gH proteins.

20 Figure 21 shows graphically the level of similarity between the DNA sequences of HSV-1 and HSV-2 in the region of the gH gene (from UWGCG program Plotsimilarity).

Figure 22 shows graphically the level of similarity between the amino acid sequences of the HSV-1 and HSV-2 gH proteins (from UWGCG program Plotsimilarity).

25

Figure 23 shows the construction of pIMMB26; two fragments from the left and right sides of the HSV2 gH gene were amplified by PCR and cloned into pUC119. The four

oligonucleotides MB57, MB58, MB59 and MB60 are shown.

Figure 24 shows the construction of pIMMB45.

Figure 25 shows construction of the first stage recombination vector pIMMB47+.

5 Figure 26 shows construction of the second stage recombination vector pIMMB46.

Figure 27 shows a restriction map analysis for recombinants HG52-D, TK minus DISC virus, TK plus DISC virus.

10 Figure 28 shows Southern blots of BamHI digestions of various viruses, probed with the right-hand flanking sequence as shown in Fig. 27. Lane 5: HG52-D virus, lane 2: TK-minus "first stage" DISC virus and lanes 3, 4, 6, 7 and 8: TK-plus "second stage" DISC viruses.

15

Herpes Simplex Virus Deleted in Glycoprotein H (gH-HSV)

Herpes simplex virus (HSV) is a large DNA virus which causes a wide range of pathogenic symptoms in man, including recurrent facial and genital lesions, and a rare though often fatal encephalitis. In general, it seems that type 1 HSV (HSV-1) seems to be particularly associated with facial lesions, whilst type 2 HSV (HSV-2) seems to be particularly associated with genital lesions. To some extent infection with HSV can be controlled by chemotherapy using the drug Acyclovir, but as yet there is no vaccine available to prevent primary infection or the consequences of this infection. Thus there is a need both for better therapeutics to treat established HSV infections and for

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25

prophylactics to prevent the establishment of HSV infection and/or its associated pathology.

5 A difficulty with vaccination against HSV is that the virus generally spreads within the body by direct transfer from cell to cell. Thus humoral immunity is unlikely to be effective, since circulating antibody can only neutralise extracellular virus. Of more importance for the control of virus infection, is cellular immunity, and so a vaccine which is capable of generating both humoral and cellular
10 immunity, but which is also safe, would be a considerable advantage.

A suitable target gene for inactivation within the HSV genome is the glycoprotein H gene (gH). The gH protein is a glycoprotein which is present on the surface of the virus
15 envelope. This protein is thought to be involved in the process of membrane fusion during entry of the virus into the infected cell. This is because temperature sensitive virus mutants with a lesion in this gene are not excreted from virus infected cells at the non-permissive temperature
20 (Desai et al., J. Gen. Virol. 69, 1147-1156, 1988). The protein is expressed late in infection, and so in its absence, a considerable amount of virus protein synthesis may still occur.

All genetic manipulation procedures are carried out
25 according to standard methods described in "Molecular Cloning", A Laboratory Manual, eds. Sambrook, Fritsch and Maniatis, Cold Spring Harbor Laboratory Press, 1989.

METHODOLOGYPREPARATION OF A DISC HSV-1A. Generation of a Cell Line Expressing the HSV-1 gH gene

5 This was carried out in accordance with the teaching of W092/05263 published on 2 April 1992 and incorporated herein by reference and also using standard procedures in the art.

B. Production of a DISC HSV Type 1 Virus Having an Interrupted gH Gene

10 This was carried out in accordance with the teaching of W092/05263 published on 2 April 1992 and incorporated herein by reference and also using standard procedures in the art.

15

C. Relevant Publications

- (i) Forrester A. et al J. Virol. 1992; 66 p341-348
- (ii) Farrell H., et al. J. Virol. 1994; 68 p927-932

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PREPARATION OF A DISC HSV-2A. The HSV2 gH Gene

25 (a) The Herpes Simplex type 2 (HSV2) gH gene is contained within two BamH1 restriction fragments of the 25766 strain of HSV2. pTW49 is the BamH1 R fragment of HSV2 strain 25766 cloned into pBR322. pTW54 is the BamH1 S Fragment of HSV2 strain 25766 cloned into pBR322. The construction of a single plasmid containing the complete gH

gene is shown in Figure 17. pTW49 was digested with BamHI and Sall, and an 870 base pair (bp) fragment isolated from an agarose gel. Similarly pTW54 was digested with BamHI and KpnI and a 2620bp fragment isolated from an agarose gel. The two fragments were ligated together with the plasmid pUC119 cut with Sall and KpnI, resulting in the plasmid pIMMB24.

(b) pIMMB24 was digested with Sall and KpnI. In addition the plasmid was digested with DraI (which cuts in the vector sequences), to aid in isolation of the 3490bp insert. The 3490bp insert containing the HSV2 sequences was purified from an agarose gel. It was then sonicated, the ends repaired using T4 DNA polymerase and Klenow, and size fractionated on an agarose gel. A fraction containing DNA molecules of approximately 300-600bp in length was ligated into M13mpl1 cut with SmaI (Amersham International UK). The ligated mixture was transformed into E.coli strain TG1, and individual plaques were picked. Single-stranded DNA was made from each plaque picked, and was sequenced using the dideoxy method of sequencing, either with Klenow enzyme or with Sequenase, and using ³⁵S dATP.

In addition to sequencing in M13 using an oligonucleotide priming from within the M13 sequences, sequence data was also obtained by sequencing directly from the pIMMB24 plasmid using oligonucleotide primers designed from sequence already obtained. In order to obtain sequence from regions flanking the gH gene, some sequence information was also obtained from the plasmid pTW49.

Because of the high G+C ratio of HSV2 DNA, there were several sequence interpretation problems due to 'compressions' on the gels. These have yet to be resolved. In a small number of places therefore, the present sequence represents the best guess as to what the correct sequence is, based on comparisons with the previously published HSV1 sequence.

(c) The sequence of HSV2 strain 25766 in the region of the gH gene is shown in Figure 18, along with a translation of the gH in single letter amino acid code. Figure 19 shows a comparison of the DNA sequence of HSV1 and HSV2 in this region. Figure 20 shows a comparison of the deduced amino acid sequences of the HSV1 and HSV2 gH proteins. At the DNA level the overall identity is 77%. At the protein level the overall identity is also 77%, with a further 9.7% of amino acids being similar in properties. The degree of sequence similarity varies to some extent along the length of the gene, as can be seen from Figure 21, which shows graphically the level of similarity. Even more marked than the variation along the gH gene is the difference in levels of identity between HSV1 and HSV2 at the DNA level between the coding and non-coding regions. As can be seen from Figure 19, the nucleotide sequence identity is higher within the coding sequence of the gH gene than it is in the intergenic regions. Figure 21 shows this in a graphical form, with the positions of the TK, gH and UL21 genes marked.

(d) The availability of nucleotide sequence data from

around the HSV-2 gH gene enables further constructs to be made eg it allows the design of recombination vectors which enables precise deletion of the gene from the viral genome. Because of the differences between HSV1 and HSV2, particularly between the genes, may not have been possible from knowledge of the HSV1 sequence alone.

Oligonucleotides MB57, MB58, MB59 and MB75 were designed to isolate and clone the regions of sequence flanking the HSV2 gH gene. As shown in Figure 23, the oligonucleotides were used in a polymerase chain reaction (PCR) to amplify fragments of DNA from either side of the gene. Restriction sites were included in the oligonucleotides so that the resultant fragments contained these sites at their ends, enabled cloning of the fragments into a suitably cut plasmid. The following oligonucleotides, based on the HSV2 sequence, were used for this purpose:

| | | |
|----|---------------|--------------------------------------|
| | | HpaI |
| 20 | Inside right | MB57 TCAGTTAACGCCTCTGTTCCCTTCCCTTC |
| | | EcoRI |
| | Outside right | MB58 TCAGAATTTCGAGCAGCTCCTCATGTTCGAC |
| | | HpaI |
| 25 | Inside left | MB75 TCAGTTAACCGTCGTCCCGGCTGCCAGTC |
| | | HindIII |
| | Outside left | MB59 TCAAAGCTTCTGCAGCGCGGCGGGAGGTGG |
| 30 | | |

The position of these oligonucleotides is also shown on Figure 19.

In accordance with the teachings made in PCT/GB91/01632 (WO 92/05263) and common general knowledge,

such a plasmid allows the skilled person to produce a defective HSV-2 virus lacking precisely the sequences for the gH gene (see below). If these same sequences are cloned into a suitable cell carrying a copy of the gH gene deleted from the HSV-2 genome, this 'complementing cell' can then support the growth of the defective HSV-2 virus by providing the gH protein. Because the sequences have been chosen so that there is no overlap between the sequences in the cell and the sequences in the virus, the possibility of the virus acquiring the gene from the cell by recombination is virtually eliminated.

B Construction of a gH Defective Type 2 Herpes Simplex Virus (DISC HSV-2)

15 Complementing Cell Lines

It was found that cells expressing the HSV-1 gH gene (F6 cells, Forrester et al, Journal of Virology, 1992, 66, p. 341-348) can support the growth of an HSV-2 virus lacking the gH gene. However two new cell lines were made. CR1 cells use the same promoter and gH gene as F6 cells, but the sequences downstream of the gene are truncated so that there is no overlap of sequences between the final DISC virus and the cell line. This is very useful since it means that homologous recombination cannot occur between the DISC virus and the cell line DNA. In the case of F6 cells and the gH-deleted virus in the Forrester paper, where there is overlap, wild-type gH-plus viruses occur by recombination at about 1 in 10⁶ viruses. Another cell line,

CR2, was also made, which expresses the gH gene from the HSV-2 strain 25766. This also supports the growth of a DISC HSV-2 and also has no overlapping sequences between the virus and the cell.

5

Polymerase Chain Reaction (PCR) of Flanking Sequences

Viral DNA is purified from virus by standard methods. Flanking sequences to either side of the gH gene are amplified by PCR using Vent DNA polymerase (New England Biolabs) which has a lower error rate than Taq DNA polymerase (see Fig. 24). The oligonucleotides used for PCR include restriction site recognition sequences, as well as the specific viral sequences (see below). Two vectors are made, one for the first stage and one for the second stage of recombination. For both vectors the right hand flanking sequences start at the same position to the right of the gH gene. The first stage vector has left hand flanking sequences that, in addition to deleting the HSV-2 gH gene, also delete the 3' portion of the viral TK gene. The second stage vector has left hand flanking sequences which restore the complete TK gene, and extend right up to the 5' end of the gH gene, as desired in the final virus.

15

20

The oligonucleotides used are as follows:

| | | |
|----|------|--|
| 25 | MB97 | HindIII TCGAAGCTTCAGGGAGTGGCGCAGC |
| | MB96 | HpaI TCAGTTAACGGACAGCATGGCCAGGTCAAG |
| 30 | MB57 | HpaI TCAGTTAACGCCTCTGTTCCCTTCCCTTC |

MB58 EcoRI
 TCAGAATTCGAGCAGCTCCTCATGTTTCGAC

5 Construction of Vectors

 The first stage recombination vector, pIMMB47+

 The two PCR fragments made by oligos MB97-MB96 and by
oligos MB57-MB58 are digested with the restriction enzymes
appropriate to the sites that have been included in the PCR
10 oligonucleotides. The MB97-MB96 fragment is digested with
HindIII and HpaI. The MB57-MB58 fragment is digested with
HpaI and EcoRI. These fragments are then ligated into the
vector pUC119 which has been digested with HindIII and
EcoRI. The resultant plasmid is called pIMMB45 (see Fig.
15 24).

 To allow for easy detection of the first stage
recombinants, the E.coli beta-galactosidase gene, under the
control of the Cytomegalovirus (CMV) immediate early
promoter is inserted into pIMMB45. The CMV promoter plus
20 beta-galactosidase gene is excised from a suitable plasmid
carrying the promoter and gene using one or more
appropriate restriction enzymes. If necessary, the ends
are filled in using the Klenow fragment of DNA polymerase.
This is the approach taken by the present applicants.
25 However alternative methodologies will be apparent to those
skilled in the art. For example, the beta-galactosidase
gene may be under the control of the SV40 promoter, in
which case, the gene and promoter can be excised from the
plasmid pCH110 (Pharmacia PL Biochemicals) using BamHI and
30 Tth111I, and the ends are filled in using the Klenow

fragment of DNA polymerase (Ecob-Prince, M.S., et al 1993 J. Gen. Virol, 74, p. 985-994). The fragment is gel-purified. The plasmid pIMMB45 is digested with HpaI, phosphatased with Calf Intestinal Alkaline Phosphatase (CIAP) to abolish self ligation, and gel-purified. The gel-purified fragments are then ligated together to produce the plasmid pIMMB47+ (see Fig. 25).

The second stage recombination vector, pIMMB46

The two PCR fragments made by oligos MB94-MB109 and by oligos MB57-MB108 are digested with the restriction enzymes appropriate to the sites that have been included in the PCR oligonucleotides. The MB94-MB109 fragment is digested with HindIII and HpaI. The MB57-MB108 fragment is digested with HpaI and EcoRI. These fragments are then ligated into the vector pUC119 which has been digested with HindIII and EcoRI. The resultant plasmid is called pIMMB46 (see Fig. 26). The oligonucleotides used are as follows:

MB108 EcoRI
TCAGAATTCGTTCCGGGAGCAGGCGTGGA

MB109
 HpaI
TCAGTTAACTGCACTAGTTTTTAATTAATACGTATGCCGTCCGTCCCGGCTGCCAGTC

Construction of Recombinant Viruses

a) First Stage.

Virus DNA was made from strain HG52-D, which is a plaque-purified isolate of the HSV-2 strain HG52. Virus DNA (2.5 µg) and pIMMB47+ plasmid DNA (0.25 µg) was transected into CRI cells using the CaPO₄ precipitation method (Chen & Okayama, Molecular and Cellular Biology, 7,

p. 2745). Recombination takes place within the cells, and a mixture of recombinant and wild type virus is produced. The mixture was plaque-purified three times on CR1 cells in the presence of acyclovir (10 µg/ml), to select for TK-

5 minus virus. A single plaque was then grown up and analysed. The virus was titrated on normal Vero cells and on CR1 cells. If the virus is a gH-deleted virus, it should only grow on CR1 cells and not on Vero cells. Table

10 1 shows that this is the case. It can be seen that the virus does not grow at all on the non-complementing Vero cells even at the highest virus concentrations, but does grow well on the CR1 complementing cell line, which expresses the HSV-1 gH gene. The virus also grows well on CR2 cells which express the HSV-2 gH gene (data not shown).

15

Table 1: growth of first stage recombinant virus on complementing (CR1) and non-complementing (Vero) cells.

| Virus dilutions | CR1 (gH+) | | | Vero | | | |
|-----------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| | 10 ⁻⁴ | 10 ⁻⁵ | 10 ⁻⁶ | 10 ⁻¹ | 10 ⁻² | 10 ⁻³ | 10 ⁻⁴ |
| Numbers of | | >350 | 174 | 22 | 0 | 0 | 0 |
| 25 0 plaques | >350 | 169 | 19 | 0 | 0 | 0 | 0 |

b) Second stage.

30

DNA was made from this TK-minus DISC virus and a recombination was carried out as above with the plasmid pIMMB46. In this case TK-plus recombinants were selected, on a gH-expressing TK-minus BHK cell line, by growth in medium containing methotrexate, thymidine, glycine,

adenosine and guanosine. Virus was harvested and grown again under selective conditions twice more before a final plaque purification was carried out on CR1. Virus was grown up and analysed by Southern blotting. Virus DNA from the original HG52-D, the TK-minus DISC virus, and the TK-plus DISC virus were digested with BamHI and separated on an agarose gel. The DNA bands were then transferred to nylon membrane by the Southern blotting method, and probed with radiolabelled fragments from the right hand flanking sequences. Fig. 27 shows the structures of these viruses, with the expected band sizes after BamHI digestion. The probe used is marked as 'R' beneath a dashed line. The probe should hybridise to a different size band in each of these viruses, as follows:

| 15 | Virus | Band size hybridising (base pairs) |
|----|-----------------------------------|---------------------------------------|
| 20 | HG52-D | 3481 |
| | TK-minus "first stage" DISC virus | 3140 |
| | TK-plus "second stage" DISC virus | 4225 |

Fig. 28 shows that this is the case. Lane 5 shows the HG52-D virus, Lane 2 contains the TK-minus "first stage" DISC virus, and lanes 3, 4, 6, 7 and 8 contain TK-plus "second stage" DISC viruses. This confirms that the DNA structure in each of these viruses is as expected.

The present application refers to certain strains of HSV-1 and HSV-2. It is not necessary that the general teaching contained herein is put into effect with precisely the mentioned strains. Strains of HSV-1 and HSV-2 having high sequence homology to one another by which the invention may be put into effect are readily available. For example, one source of HSV is the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852 USA. The following are available from ATCC under the indicated accession numbers.

| | | |
|------------------------|---|---------------------------|
| HSV-1 strain F | : | ATCC accession no. VR-733 |
| HSV-1 strain MacIntyre | : | ATCC accession no. VR-539 |
| HSV-1 strain MP | : | ATCC accession no. VR-735 |
| HSV-2 strain G | : | ATCC accession no. VR-734 |
| HSV-2 strain MS | : | ATCC accession no. VR-540 |

IN VIVO MOUSE STUDIES

PROTECTION STUDIES

The in vivo mouse ear model was used to study prophylactic effects. Equivalent doses of inactivated wild-type HSV-1 (Strain SC16 see Hill et al. J. Gen. Virol. 28, p341-353 (1975)) and DISC HSV-1 were compared for their effect on the replication of w.t. HSV-1, their ability to provide protection against w.t. HSV-1 challenge and to induce HSV-specific neutralising antibodies.

4-5 week old BALB/c mice were vaccinated with varying doses of DISC HSV-1 or inactivated virus by scarification in the left ear pinna. Virus was inactivated using β -propiolactone (for further details see W092/05263 published

on 2 April 1992 and incorporated herein by reference). The mice were challenged with 2×10^6 pfu w.t. HSV-1 (strain SC16) in the opposite ear two weeks after vaccination. The amount of virus present in that ear 5 days post challenge was assayed by plaquing on BHK cells. (See Fig. 2.)

It can be seen from Fig. 2 that vaccination with 5×10^5 and 5×10^6 pfu DISC HSV-1 (pfu measured on complementing cell line for DISC viruses) led to complete protection against replication of the challenge virus, whilst mice vaccinated with inactivated virus still had live challenge virus present.

A similar result was obtained when virus titres were assayed from the ganglia of vaccinated animals 5 days after challenge (data not shown).

SEROLOGICAL RESPONSE TO DISC HSV-1 VACCINATION

The role of antibody in protection conferred by the DISC HSV-1 vaccination was investigated. Both neutralising antibody titres and total antibody titres, as determined by ELISA, were measured.

Groups of 6 mice were vaccinated with 5×10^5 pfu of DISC HSV-1, killed DISC HSV-1, w.t. HSV-1 (strain SC16) or with PBS and serum samples taken at 2 and 14 weeks post vaccination. Neutralising antibodies were measured in the presence of complement and expressed as the inverse of the serum dilution which reduced the number of plaques by 50%. ELISA antibody titres were measured on plates coated with HSV-1 infected BHK cell lysates and titrated to endpoint. (See Fig. 3.)

It can be seen from Fig. 3 that no significant differences in antibody titres were observed between animals vaccinated with DISC HSV-1 and an equivalent amount of killed DISC HSV-1.

5

DELAYED-TYPE HYPERSENSITIVITY (DTH) RESPONSE to DISC HSV-1 VACCINATION

The importance of a DTH response in protection against herpes virus infection has been well documented. The ability of the DISC HSV-1 to raise a DTH response was investigated by vaccinating groups of mice with DISC HSV-1, killed DISC HSV-1, and live w.t. HSV-1, by scarification of the left ear pinna.

Four doses (5×10^3 , 5×10^4 , 5×10^5 and 5×10^6 pfu) of vaccine were used, and two weeks later the vaccinated animals were challenged in the opposite ear with 10^6 pfu w.t. HSV-1 (strain SC16). The DTH response at the site of challenge was assessed by measurement of ear thickness at 24 and 48 hours post challenge and expressed as the difference between the challenged and unchallenged ears. (See Fig. 4.)

It can be seen from Fig. 4 that at low vaccine doses (5×10^3 , 5×10^4 pfu), no DTH response was observed with killed DISC HSV-1, whilst a clear DTH response was demonstrated after DISC HSV-1 vaccination. At high doses (eg 5×10^6 pfu), both the DISC HSV-1 vaccine and killed DISC HSV-1 preparations induced similar DTH responses.

The DTH responses induced by different doses of the various vaccine preparations thus correlate with their

protective effect against challenge virus replication. The efficacy of vaccination with low doses of the DISC HSV-1 vaccine may therefore be due to the induction of T cell-mediated immunity.

5

DEMONSTRATION THAT DISC HSV TYPE 1 VIRUS IS CAPABLE OF GENERATING CYTOTOXIC T CELLS

Cytotoxic T cells have been shown to be involved in the protection against, and recovery from, primary HSV infection. DISC HSV-1 vaccinated mice were therefore studied for the presence of HSV-1 specific cytotoxic T cell activity.

Cytotoxic T cell activity following immunisation was generated and assayed according to standard procedures eg as exemplified in Martin, S. et al, 1988, J. Virol. 62: 2265-2273 and Gallichan, W.S. et al, J. Infect. Dis. 168: 633-629. More specifically, groups of female BALB/c mice were immunised intra-peritoneally with 2×10^7 pfu of virus (DISC HSV-1; killed DISC HSV-1; MDK a thymidine kinase negative HSV-1 strain) on day 0 and the immunisations repeated (same dose and route) after 3 weeks. A group of control mice received 0.1ml of PBS intraperitoneally at the same time points. Ten days after the second immunisation the spleens of the mice were removed and pooled for each group.

25

Spleens were also removed from unimmunised BALB/c mice for the preparation of feeder cells (16 feeder spleens being sufficient for 4 groups of six effector spleens). All subsequent steps were performed in a laminar flow hood

using aseptic technique. The spleens were passed through a sterile tea-strainer to produce a single cell suspension in RPMI 1640 medium supplemented with 10% heat inactivated foetal calf serum (effector medium). Debris was allowed to settle and the single cell suspension was transferred to a fresh container. The cell suspensions were washed twice in effector medium (1100 rpm, 10 minutes) and then passed through sterile gauze to remove all clumps. The effector spleen cell suspensions were then stored on ice until required.

Feeder spleen cells were resuspended to 1×10^7 cells/ml in effector medium and mitomycin C was added to a final concentration of 20µg/ml. The feeder cells were incubated at 37°C for 1 hour. Feeder cells were washed four-times in PBS supplemented with 1% FCS and once in PBS with no protein. Live virus (MDK) was added to the mitomycin C treated feeder cell pellet at a concentration of 3 pfu of virus per spleen cell. Following a one hour incubation at 37°C the feeder cells were washed once with effector cell medium.

Effector cells were resuspended to 5×10^6 cells/ml, whilst feeder cells were resuspended to 2.5×10^6 cells/ml. 500µl of effector cell suspension and 500µl feeder cell suspension were added to the wells of a 24 well plate. The plates were incubated in a humid atmosphere at 37°C (5% CO₂) for 4 days.

The effector and feeder cells were harvested from the 24 well plate. The cells were spun down once and the pellet resuspended in effector medium (5ml of medium per 2

plates). The cell suspension was layered onto lymphocyte separation medium and spun at 2500rpm for 20 minutes. The live effector cells were harvested from the interface and washed twice, once at 1500 rpm for 15 minutes and once at 5 1100 rpm for 10 minutes. The effector cells were finally resuspended at the required concentration in effector medium and stored on ice until required.

Labelled target cells were prepared for the cytotoxicity assay. Uninfected syngeneic A202J target 10 cells A20/2J cells were harvested from tissue culture flasks; 2×10^7 cells were added to each of 2 containers (to become infected and uninfected targets). The cells were washed with DMEM (with no additions). To the infected cells live MDK virus was added at 10pfu per cell and an 15 equivalent volume of EMEM was added to the uninfected cells. One mCi of ^{51}Cr was added to each of the universals and the cells were incubated at 37°C (in a waterbath) for 1 hour. The target cells were then washed three times (10 minutes, 1100rpm) in target medium (DMEM supplemented with 20 10% FCS) and finally resuspended to the required cell concentration in target cell medium.

Both uninfected and infected target cells were resuspended to 1×10^6 cells/ml and 1×10^5 cells/ml and 100 μl (ie to give 1×10^5 targets/well and 1×10^4 25 targets/well respectively) was plated out into the appropriate wells of a round bottomed 96 well plate. All experimental points were set up in quadruplicate. Each effector cell type was resuspended to 8×10^6 cells/ml in effector medium and two-fold dilutions were prepared.

100µl of the effector cell suspensions were added to the wells containing the labelled target cells to give 8×10^5 effector cells/well, 4×10^5 effector cells/well, 2×10^5 effector cells/well and 1×10^5 effector cells/well. Thus with 10^5 target cells per well, effector to target ratios were: 8:1, 4:1, 2:1 and 1:1. With 10^4 target cells per well the effector to target ratios were 80:1, 40:1, 20:1 and 10:1. Maximum chromium release for each target cell type was obtained by adding 100µl of 20% Triton X-100 to wells containing target cells only (ie no effectors). The spontaneous release for each target cell type was obtained by the addition of 100µl effector cell medium to wells containing target cells only.

The plates were incubated at 37°C for four hours in a humid atmosphere. After this time the plates were spun for four minutes at 1500rpm and 100µl of supernatant was removed from each of the wells. The supernatant was transferred to LP2 tubes and radioactivity contained in the tubes was then counted for 1 minute on a gamma counter. The % specific chromium release was determined using the formula

$$\% \text{ specific release} = \frac{\text{Exp. mean cpm} - \text{spon. mean cpm}}{\text{Max. mean cpm} - \text{spon. mean cpm}} \times 100$$

Exp. = Experimental

Spon. = Spontaneous

Max. = Maximum

The results are shown in Fig. 5 and Table 1

Table 1

| E:T ratio | DISC HSV-1 | Inactivated Virus | MDK | Unvaccinated |
|-----------|------------|-------------------|------|--------------|
| 8:1 | 53.9 | 1.5 | 48.3 | ND |
| 4:1 | 49.6 | 0.0 | 42.2 | 0.0 |
| 2:1 | 36.9 | 0.0 | 31.0 | 0.0 |
| 1:1 | 23.9 | 0.0 | 21.9 | 0.0 |

% HSV-1 Specific Lysis

(% lysis of HSV-infected cells minus % lysis of uninfected cells).

DISC HSV-1 vaccination induced HSV-1 specific CTL activity comparable to that produced by infection with the fully replicative MDK virus. In contrast no HSV-1 specific CTL activity was observed in mice immunised with killed DISC HSV-1 or in PBS treated animals, although some non-specific killing was observed in these animals. The reason for this is not clear, but it could represent a high level of NK cell activity.

Vaccination of mice with the DISC HSV-1 has thus been shown to induce antibody, CTL and DTH activity against HSV-1 virus antigens. The ability to activate both humoral and cell-mediated immune responses against a broad spectrum of virus proteins may explain the effectiveness of the DISC virus vaccination.

LONG-TERM PROTECTION

The in vivo mouse ear model was used to study long term prophylactic effect of DISC HSV-1

4-5 week old BALB/c mice were divided into groups containing 6 animals each. The groups were vaccinated as follows:

| | <u>Group</u> | <u>Vaccination</u> |
|----|--------------|---|
| | PBS | Mock immunisation with PBS |
| 10 | 1K | 1 immunisation with inactivated DISC HSV-1 |
| | 2K | 2 immunisations with inactivated DISC HSV-1 |
| | 1L | 1 immunisation with (live) DISC HSV-1 |
| | 2L | 2 immunisations with (live) DISC HSV-1 |
| | 1S | 1 immunisation with w.t. HSV-1 (strain SC16) |
| 15 | 2S | 2 immunisations with w.t. HSV-1 (strain SC16) |

All groups were immunised by scarification of the left ear pinna with 5×10^5 pfu on day 0 and blood samples taken on days 15, 27, 90, 152 and 218. Groups PBS, 2K, 2L and 2S received additional immunisations of PBS or 5×10^5 pfu on day 20. All groups were challenged with 5×10^5 w.t. HSV-1 (strain SC16) on day 223. The amount of virus present in the challenged ear (right) 5 days post challenge was assayed by plaquing on BHK cells. The results as depicted by Fig. 14 show that two vaccinations with DISC HSV-1 (group 2L) provides goods protection compared to inactivated DISC HSV-1 (group 2K), but that better protection was obtained with w.t. HSV-1 (strain SC16). The efficacy of vaccination with w.t. HSV-1 is of course, to be expected. However the use of normal live viruses as vaccines is generally undesirable. Fig. 15 shows the neutralising antibody titres induced by the various vaccinations. This shows that since 2 doses of DISC HSV-1

produce the same titre as two doses of the inactivated DISC HSV-1, the protective effect of DISC HSV-1 cannot be simply explained by antibody induction.

PROPHYLACTIC EFFECT OF DISC HSV-2

5 The in vivo mouse ear model was used to study the prophylactic effect of DISC HSV-2.

Six week old BALB/c mice were divided into groups. They were immunised by scarification of the left ear pinna as follows.

| 10 | <u>Group</u> | <u>Vaccination Material and Dose</u> |
|----|--------------|--|
| | 1 | 5 x 10 ² pfu live DISC HSV-2 |
| | 2 | 5 x 10 ³ pfu live DISC HSV-2 |
| | 3 | 5 x 10 ⁴ pfu live DISC HSV-2 |
| | 4 | 5 x 10 ⁵ pfu live DISC HSV-2 |
| 15 | 5 | 5 x 10 ² pfu killed DISC HSV-2 |
| | 6 | 5 x 10 ³ pfu killed DISC HSV-2 |
| | 7 | 5 x 10 ⁴ pfu killed DISC HSV-2 |
| | 8 | 5 x 10 ⁵ pfu killed DISC HSV-2 |
| | 9 | 5 x 10 ⁴ pfu w.t. HSV-2 (strain HG52) |
| 20 | 10 | 5 x 10 ⁵ pfu w.t. HSV-2 (strain HG52) |
| | 11 | PBS |

The DISC HSV-2 was a gH deletion mutant of strain HG52

25 Three weeks later, all groups were challenged by scarification of the right ear pinna with 5 x 10⁴ of w.t. HSV-2 (strain HG52).

30 The amount of virus present in the challenged ear (right) 5 days post challenge was assayed by plaquing on BHK cells (see Fig. 16). The results as depicted by the figure show that vaccination with DISC HSV-2 at doses of 5 x 10³, 5 x 10⁴ and 5 x 10⁵ pfu provides good protection against challenge with w.t. HSV-2 (strain HG52) compared to killed DISC HSV-2. However and as is to be expected, better protection was obtained with w.t. HSV-2 at doses of

5 x 10⁴ and 5 x 10⁵ pfu, but the use of normal live wild type viruses as vaccines is undesirable.

IN VIVO GUINEA PIG STUDIES

5 As mentioned earlier, HSV-2 appears to be closely associated with genital lesions. The guinea pig currently provides the best animal model for primary and recurrent genital disease in humans (Stanberry, L.R. et al. J. Inf. Dis. 1982, 146, 397-404).

10 Therefore the applicants have extended the earlier described mouse studies to the guinea pig vaginal model of HSV-2 infection which provides a useful system to assess the immunogenicity of candidate vaccines against genital HSV-2 infection in humans. It permits a comprehensive
15 assessment of primary clinical symptoms following intra-vaginal challenge with HSV-2, and also analysis of the frequency of subsequent recurrences.

 (1) Groups of 14 animals were immunised with two doses of the DISC HSV-1 vaccine (2 x 10⁷ pfu, 3 weeks apart)
20 either by non-traumatic introduction into the vagina (intra-vaginal route), or by scarification of the ear pinna (intra-epithelial route). A control group of 21 animals was vaccinated intra-vaginally with a mock virus preparation and a further group of 14 animals was
25 vaccinated intra-epithelially with two equivalent doses of β -propiolactone-inactivated w.t. HSV-1.

 Vaccinated animals were challenged 3 weeks later with 10^{5.2} pfu w.t. HSV-2 virus (strain MS) and monitored for the symptoms of primary and recurrent disease.

(a) Following w.t. HSV-2 challenge, animals were assessed daily over a two week period for symptoms of primary infection. Clinical lesions were scored as a direct numerical value, and erythema was scored on a scale of 1-5. The vaginal area was also measured as an index of oedema (data not shown). The results are shown in Figs. 6 and 7. Points on the graphs represent mean erythema score per animal per day (Fig. 6) and mean total lesion score per day per animal (Fig. 7).

The results show that intra-epithelial and intra-vaginal vaccination with the DISC HSV-1 both provided a high degree of protection against the primary symptoms of HSV-2 infection. Surprisingly, inactivated HSV-1 administered by the intra-epithelial route also provided substantial protection, though apparently less than that afforded by the DISC virus vaccine.

(b) Daily vaginal swabs were taken from all animals over a 12 day period post-challenge and virus titres determined by plaquing on Vero cells in order to monitor growth of the challenge virus in the vagina. The results as depicted in Fig. 8 shows that infection virus titres in mock-vaccinated animals rose to a maximum of 3×10^4 at day 2 post challenge, and could be detected until day 10. By contrast, virus titres in the vaccinated animals declined steadily from day 1, and were undetectable by day 7. No significant difference was observed between the groups immunised with the DISC HSV-1 or the inactivated virus preparation.

(c) Following HSV-2 challenge, animals which had fully recovered from the acute phase of disease by 28 days were monitored daily for a further 100 days for the recurrence of disease. Numbers of animals in each group were: DISC/Intra-vaginal -14; DISC/Intra-epithelial -12; Inactivated/Intra-epithelial -14; Mock/Intra-vaginal -12. Clinical lesions were scored as a direct numerical value, and erythema was scored on a scale of 1-5. The results are shown in Figs. 9a and 9b. Points on the graphs represent the cumulative totals of mean values per day per animal.

The results show that animals vaccinated with the DISC HSV-1 by the intra-vaginal route showed approximately a 50% reduction in the number of recurrent HSV-2 lesions occurring over the 100 day follow-up period. Intra-epithelial vaccination with DISC HSV-1 and inactivated virus also resulted in a reduction of recurrent lesions, but to a lesser extent.

(2) The following experiment was also designed to assess the immunogenicity of candidate DISC vaccines based on HSV-1 against genital HSV-2 infection. The experiment was designed to compare different vaccination routes (per vaginum, oral and nasal ie different mucosal surfaces) and different doses of either DISC HSV-1 or inactivated HSV-1 in the guinea pig.

Materials and Methods

Virus.

(i) DISC HSV-1 was propagated on Vero cells (F6)

which had been transfected with the HSV-1 gH gene as described previously in W092/05263 published on 2 April 1992. Briefly, confluent monolayers of F6 cells were infected with DISC HSV-1 at a multiplicity of 0.1 pfu per cell and harvested when 90-100% cpe was observed. Cells were harvested with a cell scraper, pelleted by centrifugation and the pellet resuspended in a small volume of Eagles Minimum Essential Medium (EMEM). The suspension was sonicated for 1 minute and stored in aliquots at -70°C. Virus titres were determined on F6 cells.

(ii) DISC HSV-1 was inactivated by the addition of β -propiolactone at a concentration of 0.05% for one hour at room temperature. Inactivation was checked by adding the virus to F6 cells.

(iii) HSV-2 strain MS was propagated and titred on Vero cells in the same manner as DISC HSV-1 as described above.

Animals

Female Dunkin-Hartley guinea-pigs (300-350g) were obtained from Davis Hall, Darley Oaks Farms, Newchurch, Nr. Burton-on-Trent.

Experimental design

Groups of 12 animals were immunised with two doses of 8×10^6 pfu DISC HSV-1 or with equivalent doses of inactivated DISC HSV-1, on days 1 and 17 of the experiment. Immunisation was performed with either 0.05 ml of virus intravaginally, with 0.2 ml of virus intranasally or with 0.2 ml virus orally. A control group of 12 animals was vaccinated intravaginally with a mock preparation of virus

consisting of sonicated Vero cells. All groups were challenged intravaginally on day 34 with $10^{5.2}$ pfu HSV-2 (strain MS) and the experiment blinded by randomisation of the cages by an independent worker. For a period of 11 days following challenge, animals were monitored for the symptoms of primary disease. Clinical observations were scored as the number of lesions present in the vaginal area and the presence of erythema (scored on a scale of 1-5). In addition, daily vaginal swabs were taken from all animals over a 12 day period post challenge and virus titres were determined by plaquing on Vero cells in order to monitor growth of the challenge virus in the vagina.

Statistical methods

Differences in group clinical scores were tested for significance using the Mann-Whitney U test. Values of $p < 0.1$ were considered significant.

Results

Clinical disease profile.

The mean lesion score per animal, the mean erythema score and the effect of vaccination on post challenge virus replication for each of the immunisation groups are shown in Figs 10, 11 and 12 respectively. As compared to mock vaccinated animals, vaccination with DISC HSV-1 by the intravaginal route provided a high degree of protection from primary symptoms of infection. In contrast, vaccination with inactivated DISC HSV-1 at an equivalent dose did not lead to any significant protection.

Intranasal immunisation with DISC HSV-1 resulted in an even higher degree of protection than intravaginal

vaccination. This was particularly apparent when looking at the number of days with severe disease, as defined by a lesion score of 6 or more (see table 2). Inactivated DISC HSV-1 gave some protection via the intranasal route, but it was not as effective as vaccination with DISC HSV-1.

Vaccination via the oral route also led to protection, but to a lesser degree than intranasal or intravaginal vaccination. Again vaccination with DISC HSV-1 virus protected more efficiently than vaccination with inactivated DISC HSV-1.

Table 2

INCIDENCE OF PRIMARY DISEASE SYMPTOMS

| Immunisation with | Any disease symptoms (% of animals) | Lesion score >5 (% of animals) | Duration of disease (mean no. days) | Disease ongoing on day 11 (% of animals) |
|-------------------------|-------------------------------------|--------------------------------|-------------------------------------|--|
| mock | 92 | 75 | 6.8 | 75 |
| DISC HSV-1 i.vag | 33 | 17 | 4.5 | 8 |
| HSV-1 inactivated i.vag | 92 | 67 | 6.2 | 83 |
| DISC HSV-1 i.nas | 33 | 0 | 2.3 | 0 |
| HSV-1 inactivated i.nas | 67 | 17 | 6.3 | 42 |
| DISC HSV-1 oral | 90 | 20 | 4.1 | 20 |
| HSV-1 inactivated oral | 91 | 36 | 5.8 | 64 |

Thus the following conclusions can be drawn from this experiment with the in vivo guinea pig model.

- 5 A. Vaccination with DISC HSV-1 via the intravaginal and intranasal routes led to a high degree of protection from acute disease symptoms following a challenge with HSV-2.
- 10 B. Intranasal administration of DISC HSV-1 gave the highest degree of protection when considering the number of days of severe disease (as defined by the presence of 6 or more lesions).
- 15 C. Intravaginal vaccination with inactivated virus resulted in clinical disease symptoms similar to those observed in mock-infected guinea-pigs. Intranasal vaccination with inactivated DISC HSV-1 gave a significant degree of protection, but not as high as DISC HSV-1 vaccination via this route.
- 20 D. A significant difference was observed between disease symptoms in animals vaccinated orally with DISC HSV-1 and mock-infected animals. However, this degree of protection was less than that observed in animals vaccinated with DISC HSV-1 via the intranasal or intravaginal route.
- 25 E. Symptoms in animals vaccinated orally with inactivated DISC HSV-1 were not significantly different from those in the mock-infected group.
- F. The data on shed virus is interesting. Surprisingly the per vaginum vaccination route resulted in significantly lower levels of recovered

virus following the challenge dose. This may be due to local antibody production.

(3) The following experiment was designed to
5 investigate HSV-2 induced recurrent disease following
therapeutic vaccination.

This was of interest as it has previously been shown
that therapeutic administration of certain recombinant HSV-
2 antigens, together with adjuvant, can decrease the
10 frequency of subsequent recurrences. (Stanberry, L.R. et
al. J. Inf. Dis. 1988; 157, p156-163; Stanberry, L.R. et
al. J. Gen. Virol. 1989a; 70 p3177-3185; Ho, R.J.Y. et al,
J. Virol. 1989; 63p 2951-2958).

Accordingly 21 animals which had recovered fully from
15 primary HSV-2 disease four weeks after challenge were
randomised into three groups, and treated with live DISC
HSV-1 intravaginally (10 animals), or intra-epithelially
(11 animals). A group of 12 animals, which had previously
acted as controls for prophylactic vaccination' (see (2)
20 above) and which had also recovered fully from primary
disease were treated with an equivalent mock preparation
(12 animals). The animals were given further identical
treatments 24 and 48 days later. The frequency of
recurrent disease was monitored from the day of first
25 treatment for a further 100 days, and the cumulative
results are shown in Fig. 13 and summarised in Table 3
below.

Table 3 - Effect of therapeutic vaccination on recurrent disease

| | Mock | | DISC HSV-1 Intra-epithelial | | DISC HSV-1 Intra-vaginal | |
|---|-------|-----------|--------------------------------|-----------|-----------------------------|-----------|
| | Total | % of Mock | Total | % of Mock | Total | % of Mock |
| 1 Mean total disease/days per animal | 9.41 | 100 | 6.90 | 73 | 7.32 | 78 |
| 2 Mean total episodes per animal | 6.27 | 100 | 4.67 | 74 | 5.10 | 81 |
| 3 Disease incidence | 12/12 | 100 | 9/11 | 82 | 10/10 | 100 |
| 4 Severity per episode | 3.21 | 100 | 3.00 | 93 | 2.86 | 89 |
| Mean duration of episode (days) | 1.49 | | 1.27 | | 1.38 | |

1 Total number of days where disease was observed (either lesions or erythema) over the whole observation period (100 days from 1 month after challenge with HSV-2)

2 Total of days disease episodes over the whole observation period (episode length defined as period between two consecutive disease-free days)

3 Proportion of animals showing any lesion or erythema score during whole observation period

4 Total sum of erythema scores and lesion numbers over the whole observation period divided by number of episodes observed

It can be seen that each of the groups treated with DISC HSV-1 appeared to experience a modest reduction (about 25%) in the overall number of disease/days and episodes especially over the 50 day period following second vaccination.

Sera were collected from these animals at the end of the 100 day observation period. The ELISA and NT antibody titres in the sera were not significantly higher than those recorded post-challenge but before therapeutic treatment and there were no significant differences in titres between the mock-treatment group and the DISC HSV-1 treated groups.

Thus therapeutic administration of DISC HSV-1 virus either intra-vaginally or intra-epithelially resulted in an apparent reduction (20-25%) in the frequency of recurrence compared with mock-treated animals.

(4) The following experiment was designed to investigate the therapeutic value of a DISC virus based on HSV-2. A DISC HSV-2 (strain HG 52) having a deletion of the gH gene was made as described earlier and in accordance with the general teaching of W092/05263 published on 2 April 1992 and incorporated herein by reference and also using standard procedures in the art. The DISC version of the strain was grown in Vero cells transfected with the HSV-2 gH gene also in accordance with the teaching of W092/05263.

The experiment was a head to head comparison of DISC HSV-1 with DISC HSV-2 in female 350-400 gms guinea-pigs.

Guinea-pigs were divided into three groups. All guinea-pigs were infected with $10^{5.8}$ pfu HSV-2 strain MS. Four weeks were then allowed for the primary disease to have both developed and resolved and for recurrences to have started. The animals were then treated. A first group of 15 animals was treated intravaginally with a mock preparation of virus consisting of sonicated Vero cells. A second group of 13 animals was treated intravaginally with 10^7 pfu DISC HSV-1. A third group of 14 animals was treated intravaginally with 10^7 pfu DISC HSV-2. Treatment was repeated in 14 days.

The results are shown in Table 4. Days 1-13 covers the period between the two treatments. Days 14-27 covers the two week period subsequent to the second treatment. Days 1-27 covers the complete period.

As shown by the results, it appears that treatment with DISC HSV-2 was effective in alleviating symptoms caused by infection with HSV-2 strain MS. Treatment with DISC HSV-2 was more effective than treatment with DISC HSV-1.

Table 4

| Group | Erythema scores | | | Lesions scores | | | Disease Days | | |
|------------|-----------------|------------|-----------|----------------|------------|-----------|--------------|------------|-----------|
| | Total | Per animal | % of Mock | Total | Per animal | % of Mock | Total | Per animal | % of Mock |
| Days 1-13 | | | | | | | | | |
| Mock | 38 | 2.53 | 100 | 66 | 4.40 | 100 | 42 | 2.80 | 100 |
| DISC HSV-1 | 34 | 2.62 | 103 | 48 | 3.69 | 84 | 34 | 2.62 | 93 |
| DISC HSV-2 | 22 | 1.57 | 62 | 40 | 2.86 | 65 | 26 | 1.86 | 66 |
| Days 14-27 | | | | | | | | | |
| Mock | 13 | 0.87 | 100 | 23 | 1.53 | 100 | 17 | 1.13 | 100 |
| DISC HSV-1 | 9 | 0.69 | 80 | 14 | 1.08 | 70 | 11 | 0.85 | 75 |
| DISC HSV-2 | 2 | 0.14 | 16 | 3 | 0.21 | 14 | 3 | 0.21 | 19 |
| Days 1-27 | | | | | | | | | |
| Mock | 51 | 3.40 | 100 | 89 | 5.93 | 100 | 59 | 3.93 | 100 |
| DISC HSV-1 | 43 | 3.31 | 97 | 62 | 4.77 | 80 | 45 | 3.46 | 88 |
| DISC HSV-2 | 24 | 1.71 | 50 | 43 | 3.07 | 52 | 29 | 2.07 | 53 |

CLAIMS

1. A pharmaceutical which comprises a mutant non-retroviral virus whose genome is defective in respect of a
5 gene essential for the production of infectious virus such that the virus can infect normal cells and undergo replication and expression of viral antigen genes in those cells but cannot produce normal infectious virus, for prophylactic or therapeutic use in generating an immune
10 response in a subject infected therewith.
2. A pharmaceutical according to claim 1, wherein the defect allows the production and release from the cells of non-infectious viral particles.
15
3. A pharmaceutical which comprises a mutant non-retroviral virus whose genome is defective in respect of a gene essential for the production of infectious virus such that the virus can infect normal cells and replicate
20 therein to give rise to the production and release from the cells of non-infectious viral particles.
4. A pharmaceutical according to any one of the preceding claims which is a vaccine capable of protecting a patient
25 immunised therewith against infection or the consequences of infection with a non-retroviral virus.
5. A pharmaceutical according to any one of the preceding claims which is a therapeutic capable of treating a patient

with an established non-retroviral virus infection.

6. A pharmaceutical according to claim 4 or claim 5 which is capable of protecting against infection or the
5 consequences of infection, or treating a patient for infection by the corresponding wild-type virus.

7. An epithelially-administrable pharmaceutical according to according to any one of claims 1 to 6 which comprises an
10 excipient for epithelial administrations.

8. A nasally-administrable pharmaceutical according to any one of claims 1 to 6 which comprises an excipient for nasal administration.

15 9. A vaginally-administrable pharmaceutical according to any one of claims 1 to 6 which comprises an excipient for vaginal administration.

20 10. An orally-administrable pharmaceutical according to any one of claims 1 to 6 which comprises an excipient for oral administration.

25 11. A pharmaceutical according to any one of claims 1 to 10 wherein the mutant is from a double-stranded DNA virus.

12. A pharmaceutical according to claim 11 wherein the mutant is from a herpes virus.

13. A pharmaceutical according to claim 12 wherein the mutant is from herpes simplex virus (HSV).

5 14. A pharmaceutical according to claim 13 wherein the mutant is a type-1 HSV.

15. A pharmaceutical according to claim 13 wherein the mutant is a type-2 HSV.

10 16. A pharmaceutical according to claim 14 or claim 15 wherein the defect is in the glycoprotein gH gene.

15 17. Use of a mutant type-1 HSV whose genome is defective in respect of a gene essential for the production of infectious HSV-1 such that the virus can infect normal cells and undergo replication and expression of viral antigen genes in those cells but cannot produce normal infectious virus, for preparation of a pharmaceutical for prophylactic or therapeutic use in generating an immune
20 response in a subject against type-2 HSV infection.

18. Use according to claim 17 wherein the pharmaceutical is for epithelial administration.

25 19. Use according to claim 17 wherein the pharmaceutical is for vaginal administration.

20. Use according to claim 17 wherein the pharmaceutical is for nasal administration.

21. Use according to claim 17 wherein the pharmaceutical is for oral administration.

5 22. An assembly comprising a pharmaceutical according to any one of claims 1 to 16 in a container with printed instructions on or accompanying the container concerning the administration of the pharmaceutical to a patient to protect against or treat conditions caused by infection with a non-retroviral virus.

10

23. An assembly according to claim 22 wherein the non-retroviral virus is a double-stranded DNA virus.

15 24. An assembly according to claim 23 wherein the double-stranded DNA virus is a herpes virus.

25. An assembly according to claim 24 wherein the herpes virus is a herpes simplex virus (HSV).

20 26. An assembly according to claim 25 wherein the HSV is type 1.

27. An assembly according to claim 26 wherein the HSV is type 2.

25

28. An assembly according to claim 26 wherein the printed instructions concern protection against or treatment of facial lesions.

29. An assembly according to claim 27 wherein the printed instructions concern protection against or treatment of genital lesions.

5 30. Use of a pharmaceutical according to any one of claims 1 to 16 to generate an anti-non-retroviral virus immune response in a patient for prophylactic or therapeutic purposes.

10 31. Use according to claim 30 wherein the non-retroviral virus is a double-stranded DNA virus.

32. Use according to claim 31 wherein the double-stranded DNA virus is a herpes virus.

15 33. Use according to claim 32 wherein the herpes virus is a herpes simplex virus (HSV).

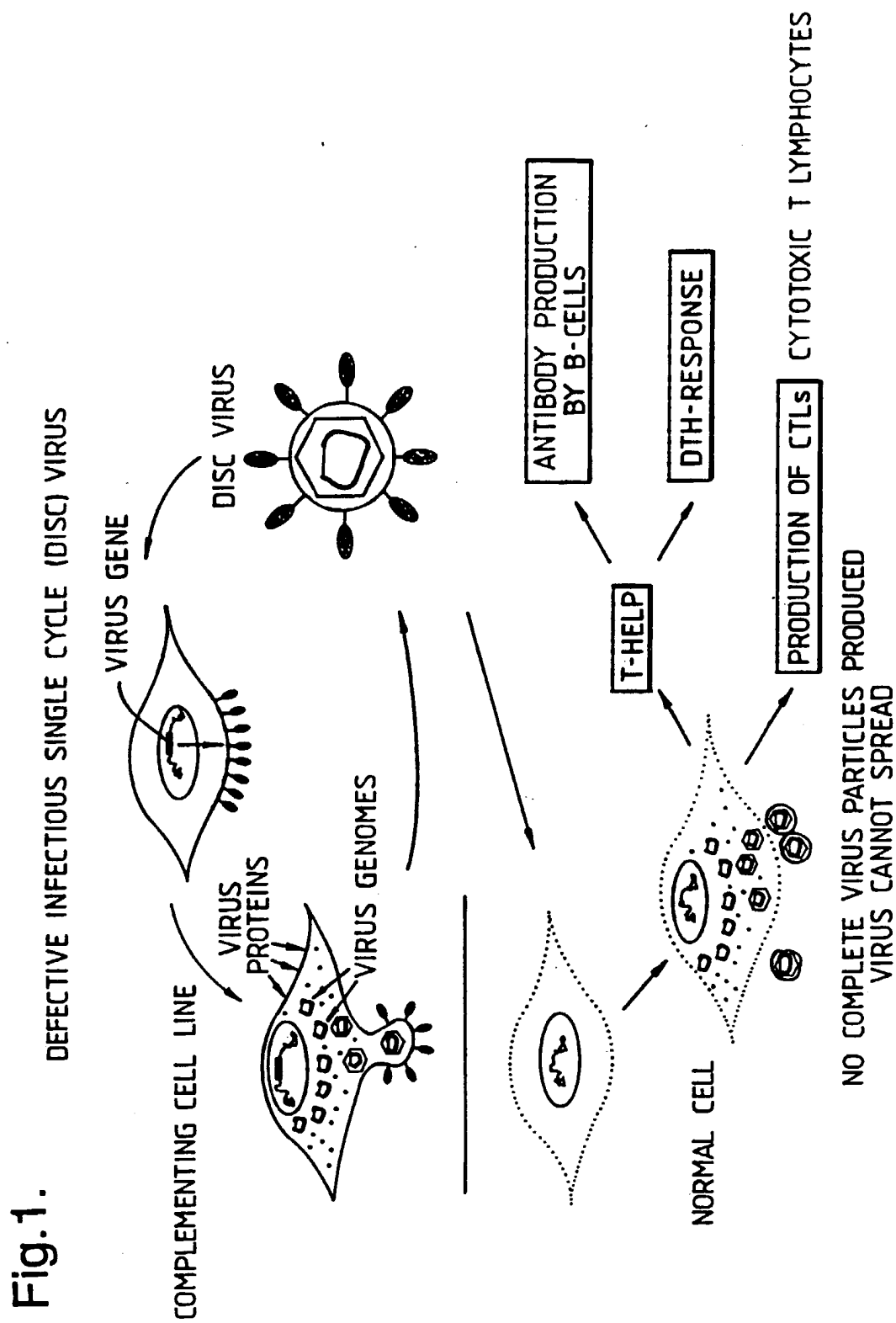
34. Use according to claim 33 wherein the HSV is type 1.

20 35. Use according to claim 34 wherein the HSV is type 2.

36. Use according to claim 34 for the prophylaxis or treatment of facial lesions in a patient.

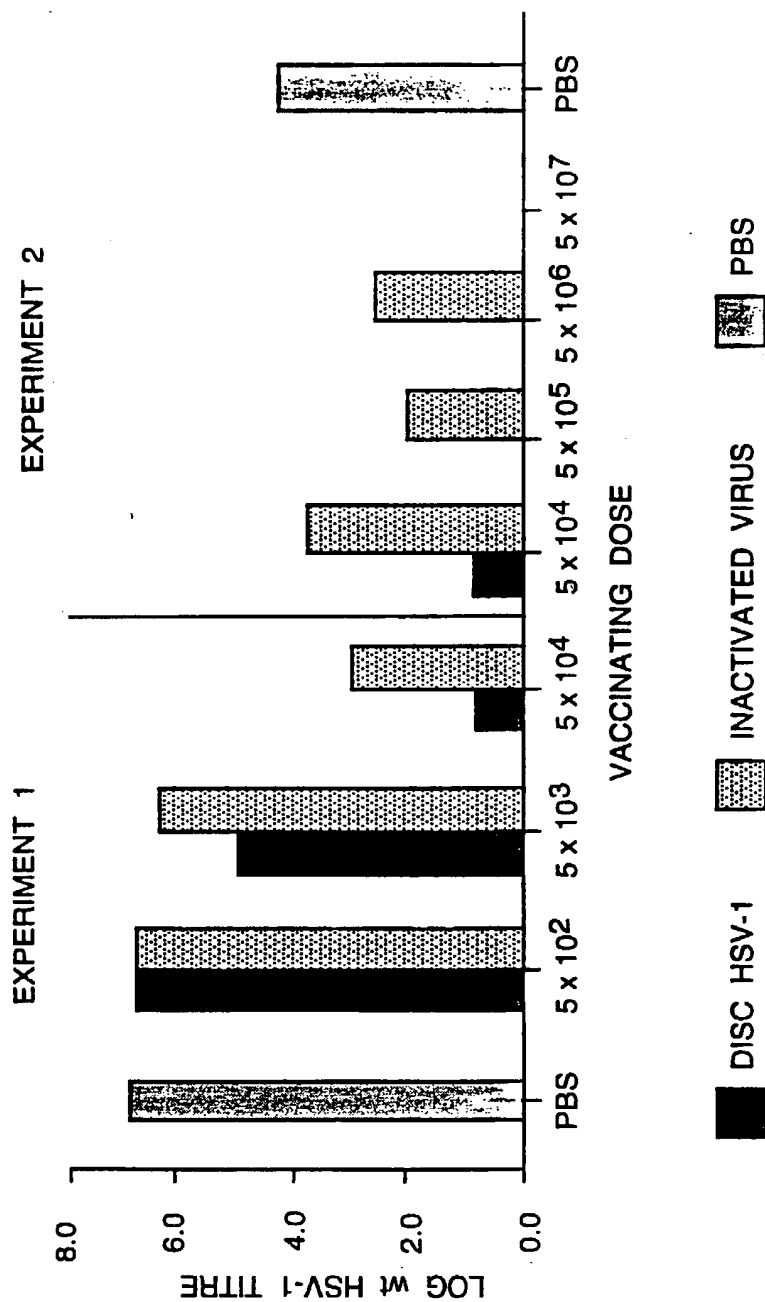
25 37. Use according to claim 35 for the prophylaxis or treatment of genital lesions in a patient.

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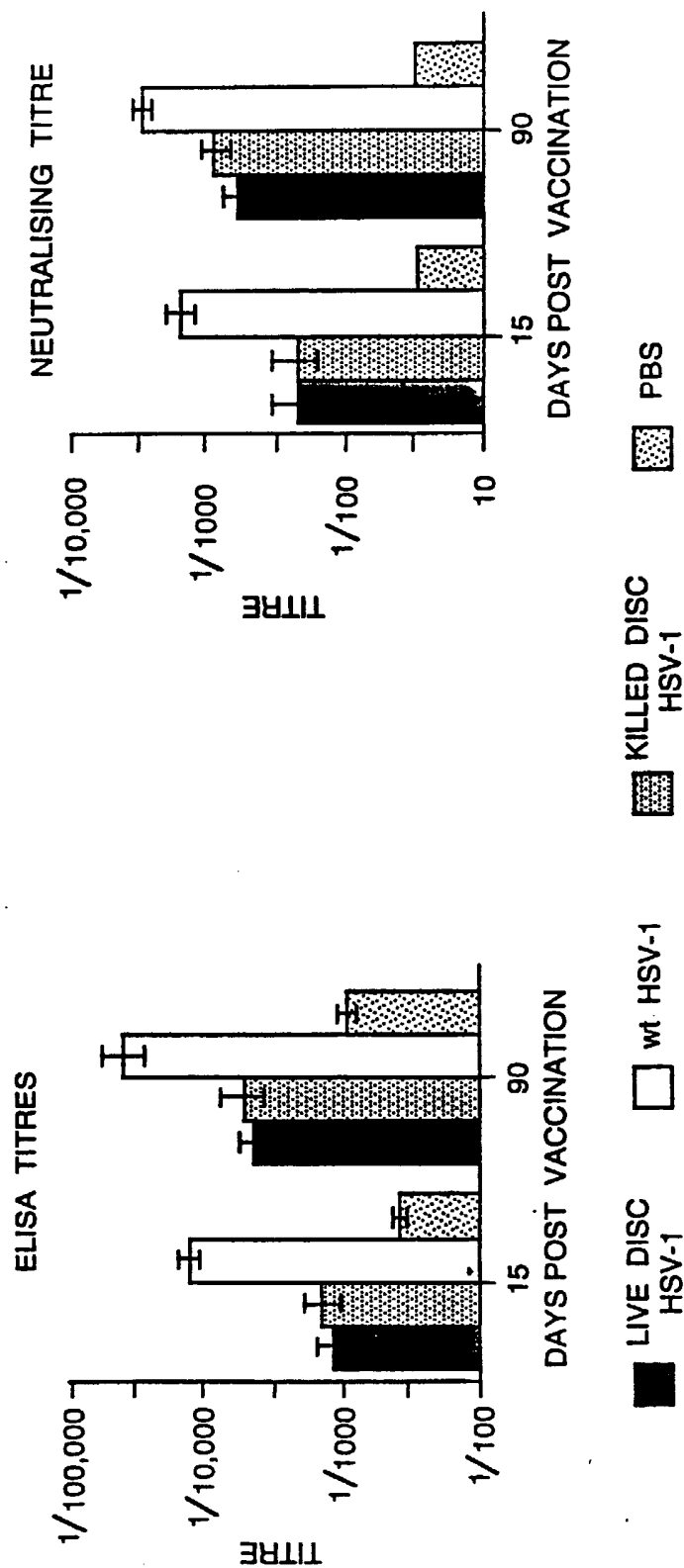
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Fig.2

EAR VIRUS TITRES-FIVE DAYS POST CHALLENGE WITH 2 x 10⁶ pfu SC16

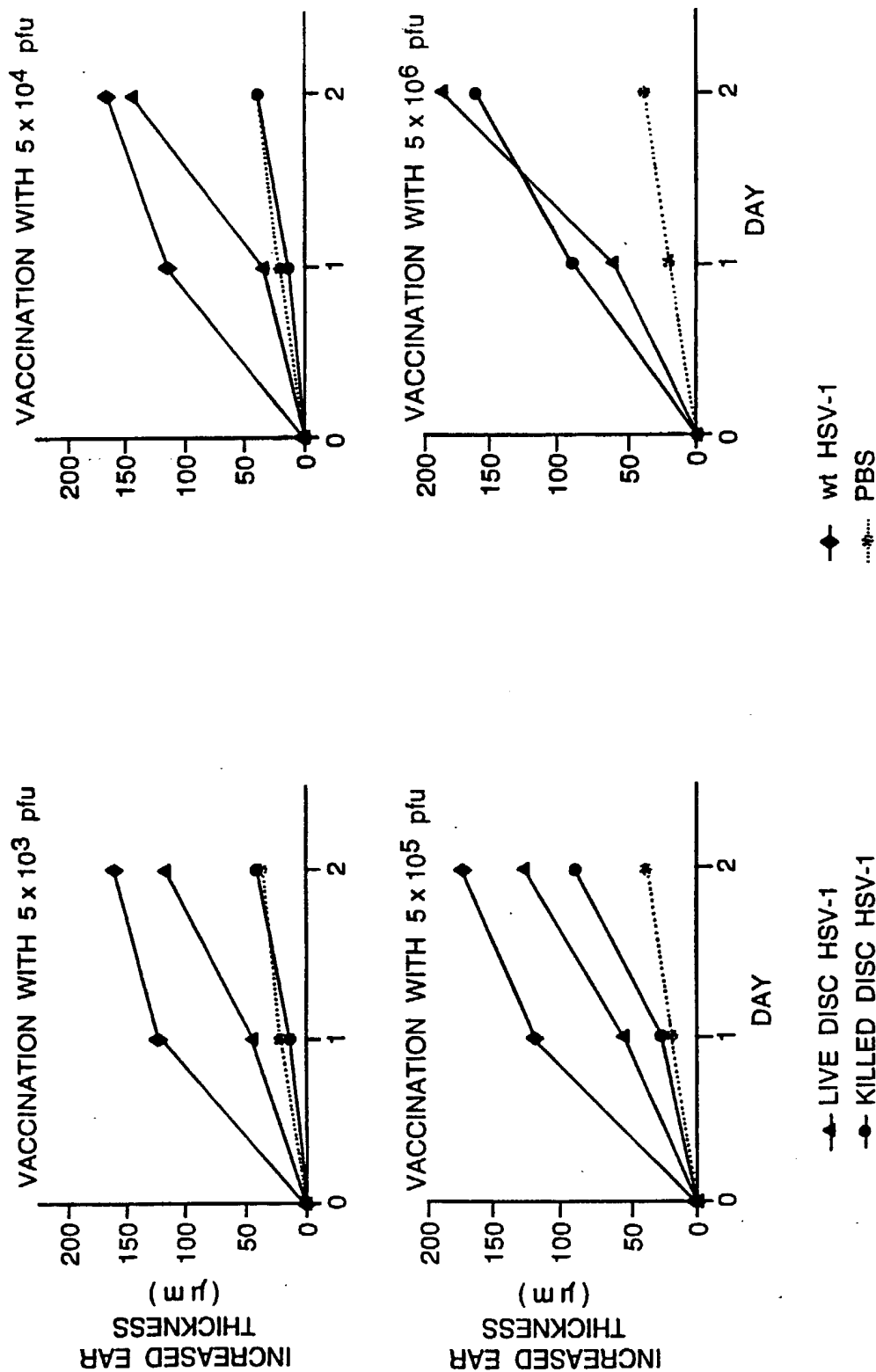
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Fig.3

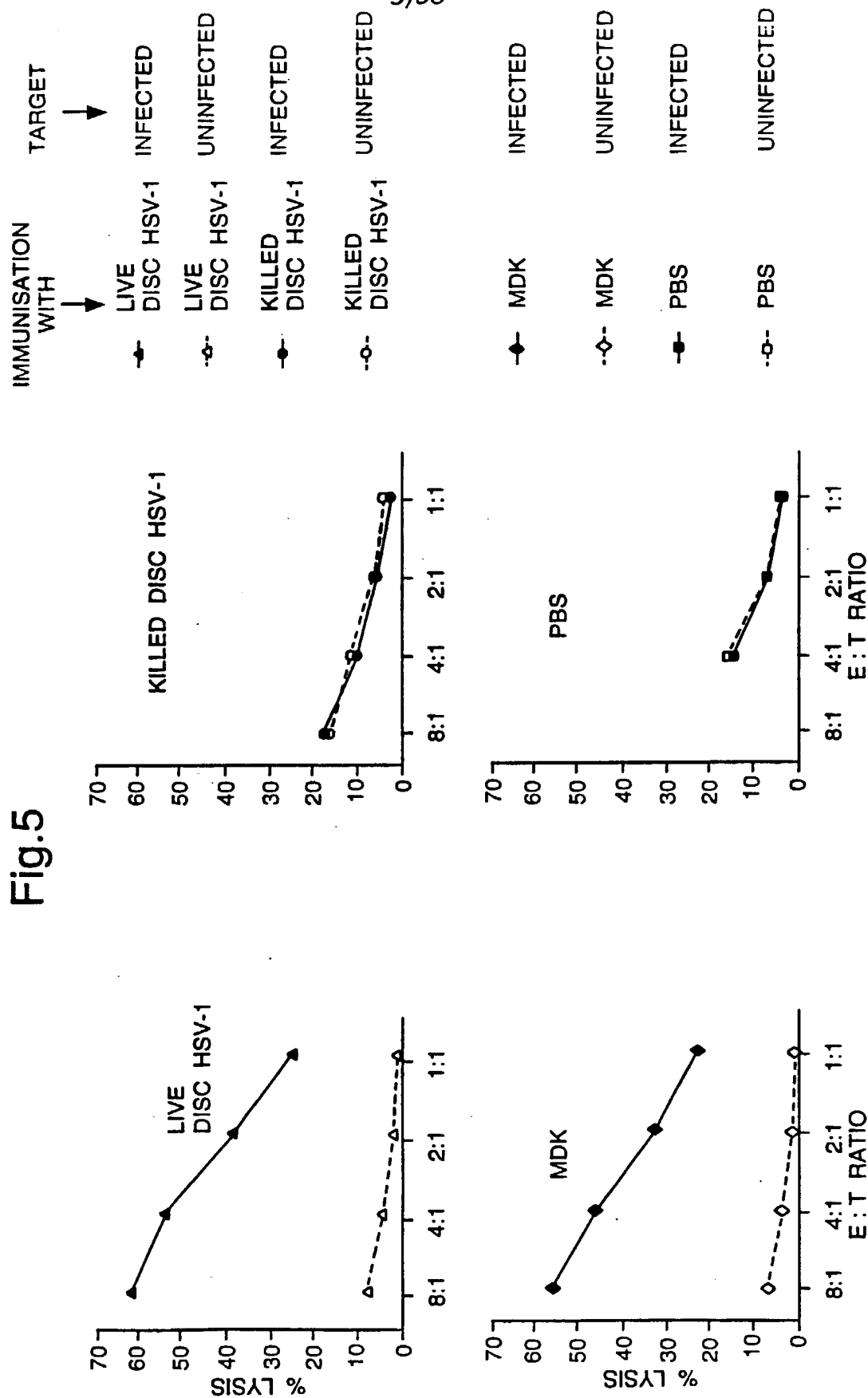


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Fig.4



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Fig.6

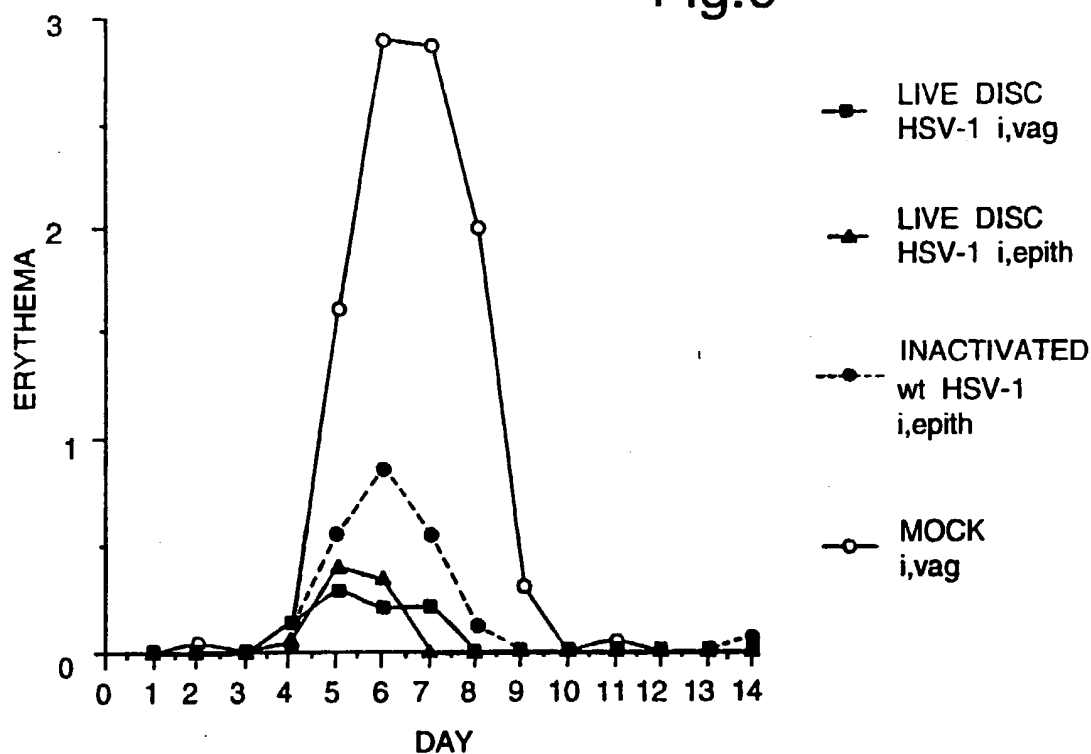
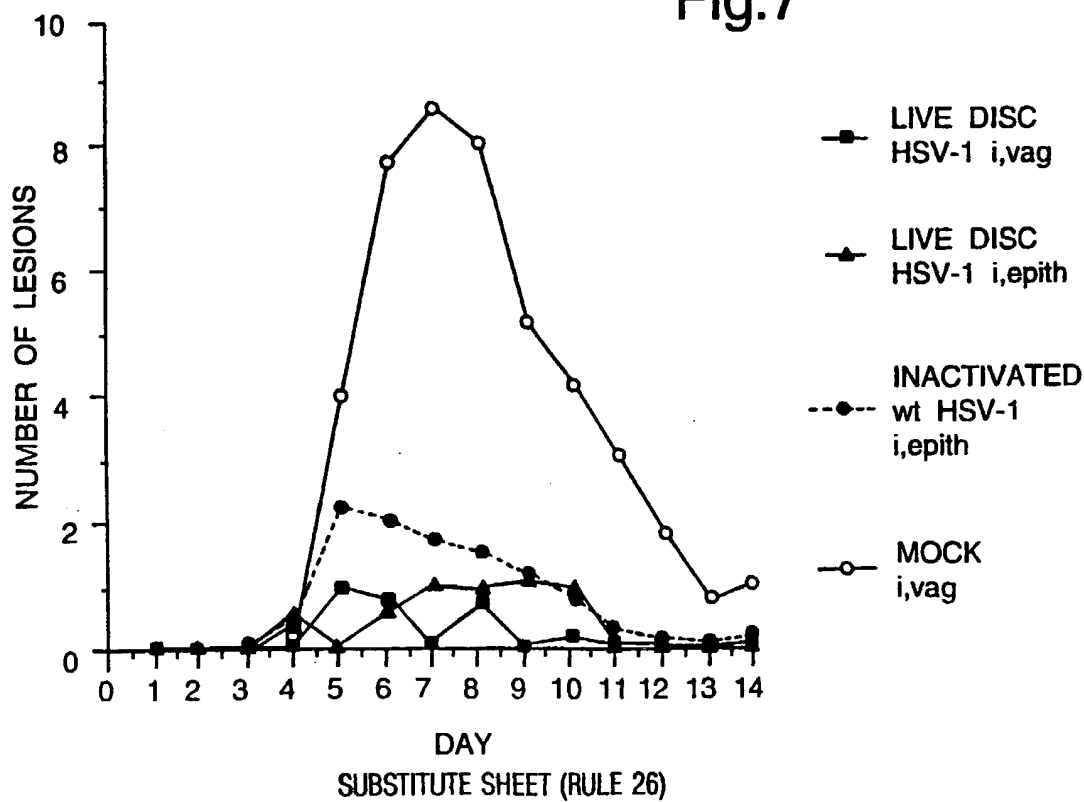


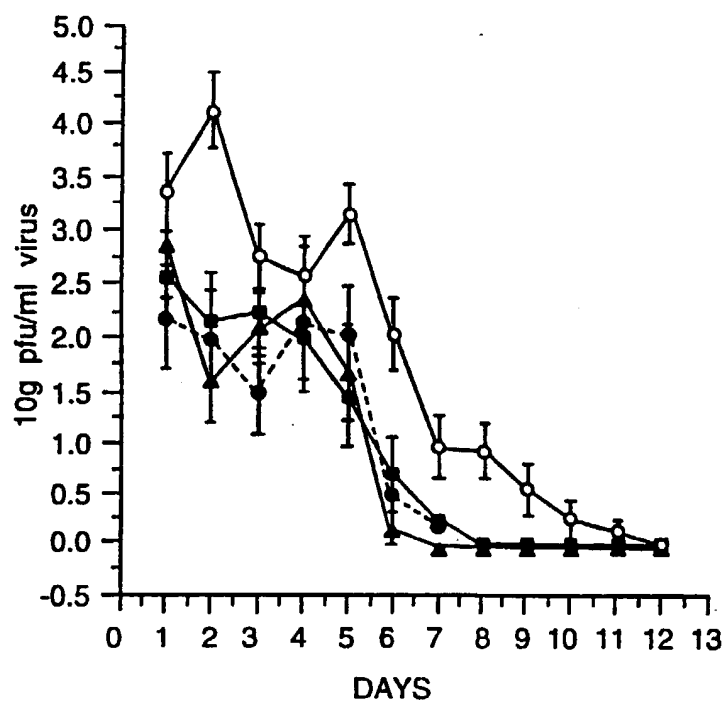
Fig.7



SUBSTITUTE SHEET (RULE 26)

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Fig.8



- LIVE DISC HSV-1 i,vag
- ▲— LIVE DISC HSV-1 i,epith
- INACTIVATED wt HSV-1 i,epith
- MOCK i,vag

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Fig.9a

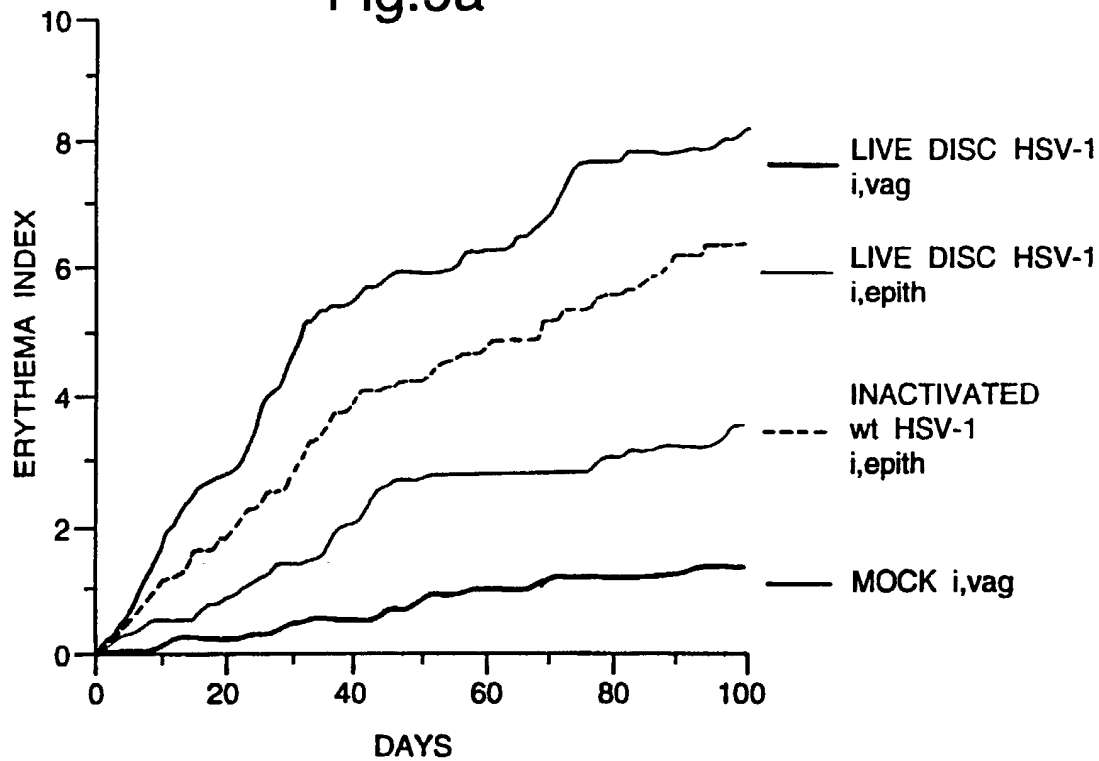
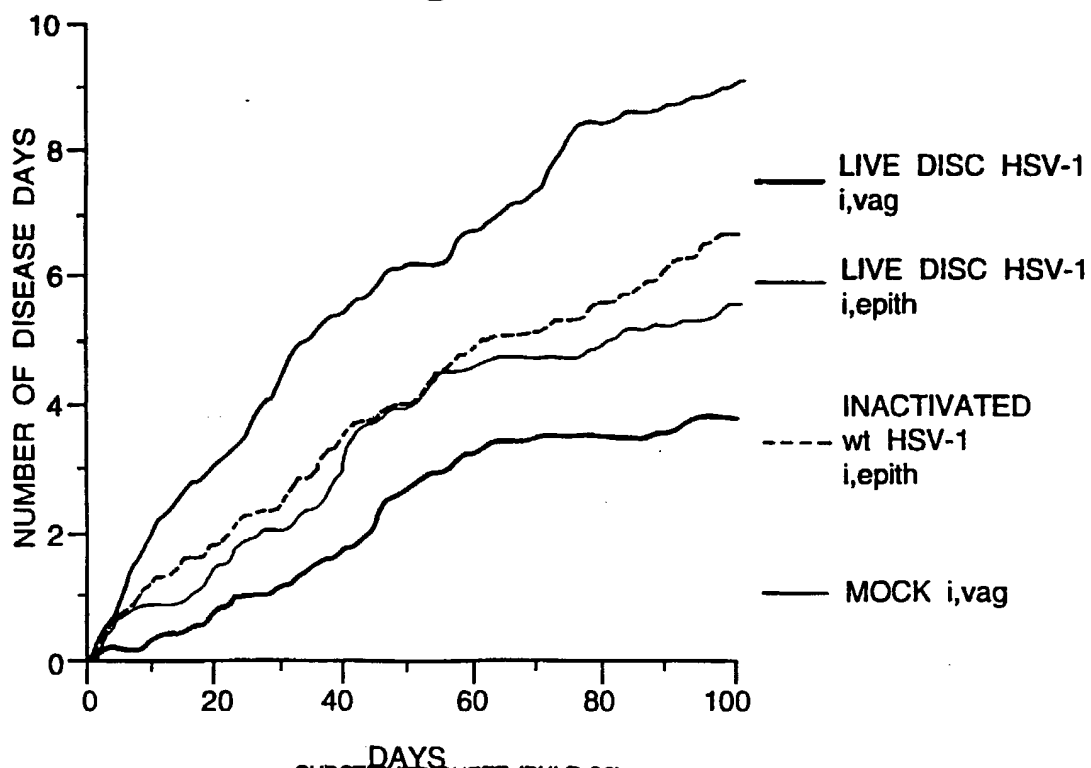
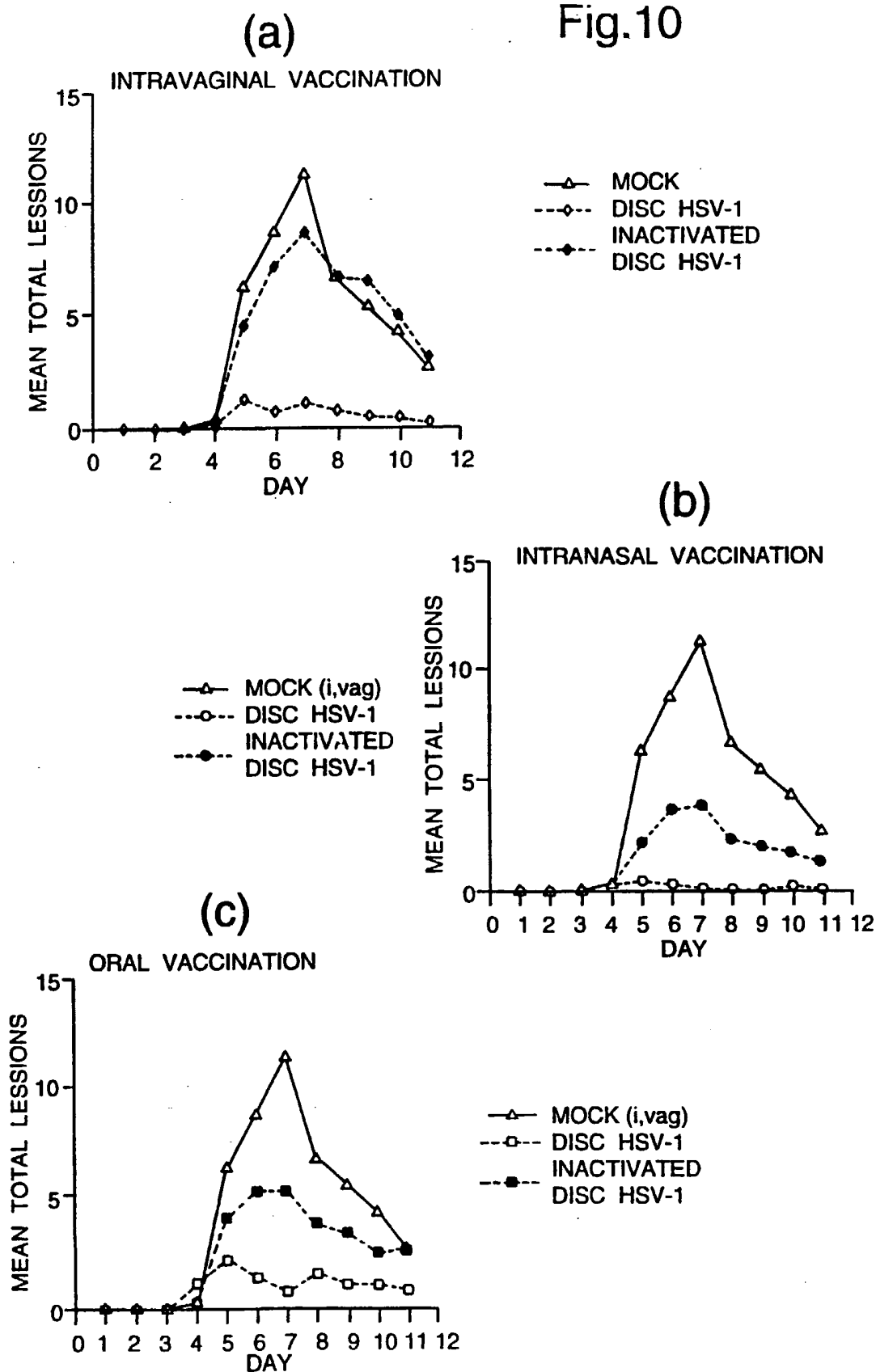


Fig.9b



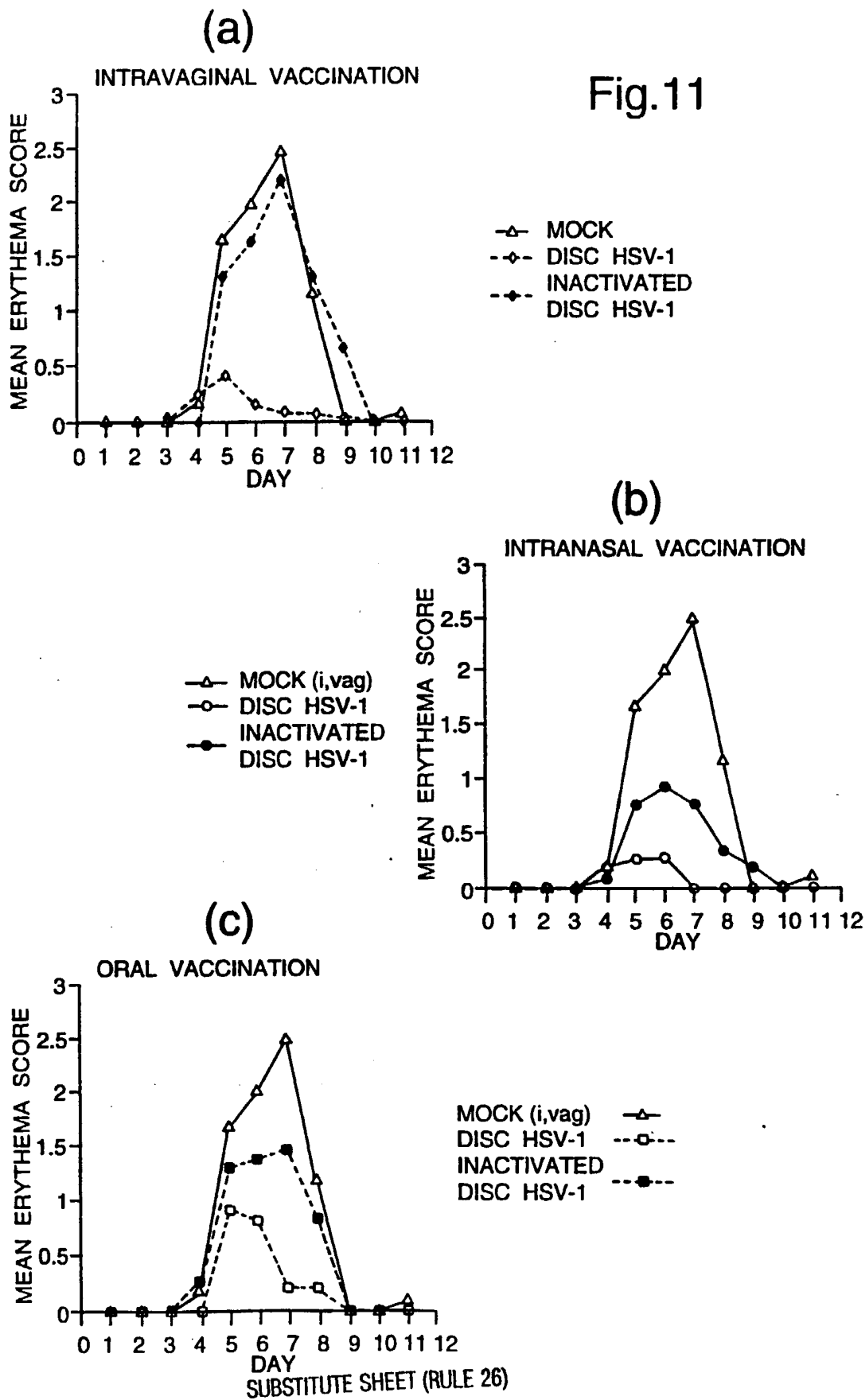
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Fig.10



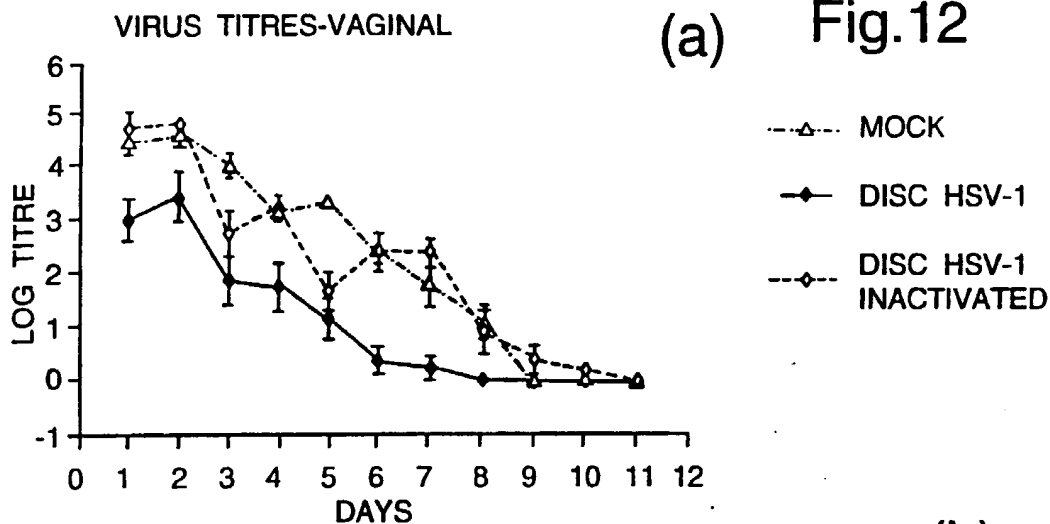
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Fig.11

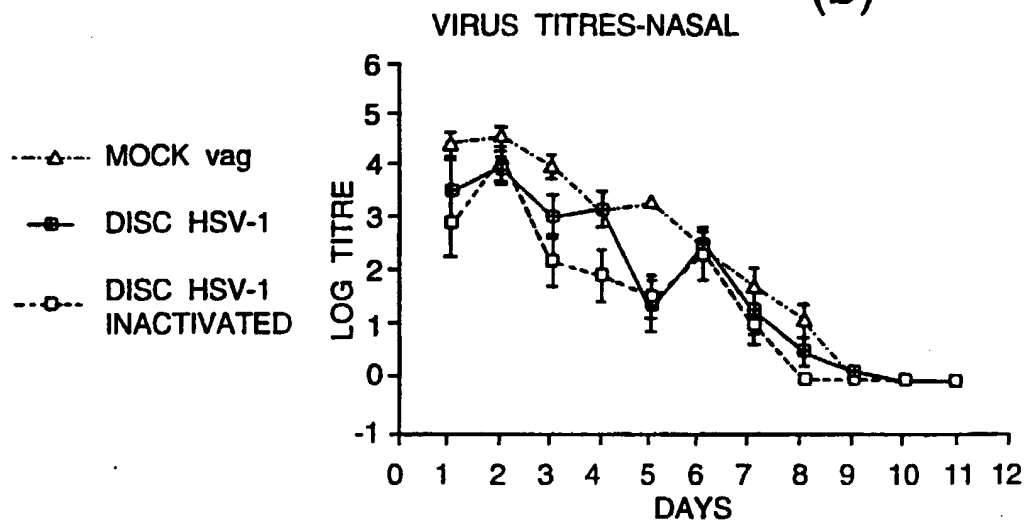


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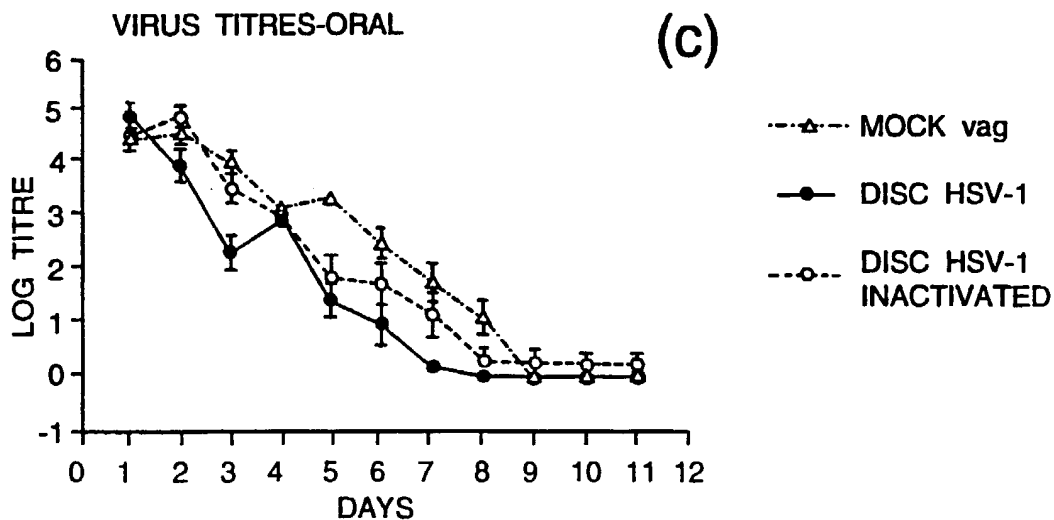
(a) Fig.12



(b)

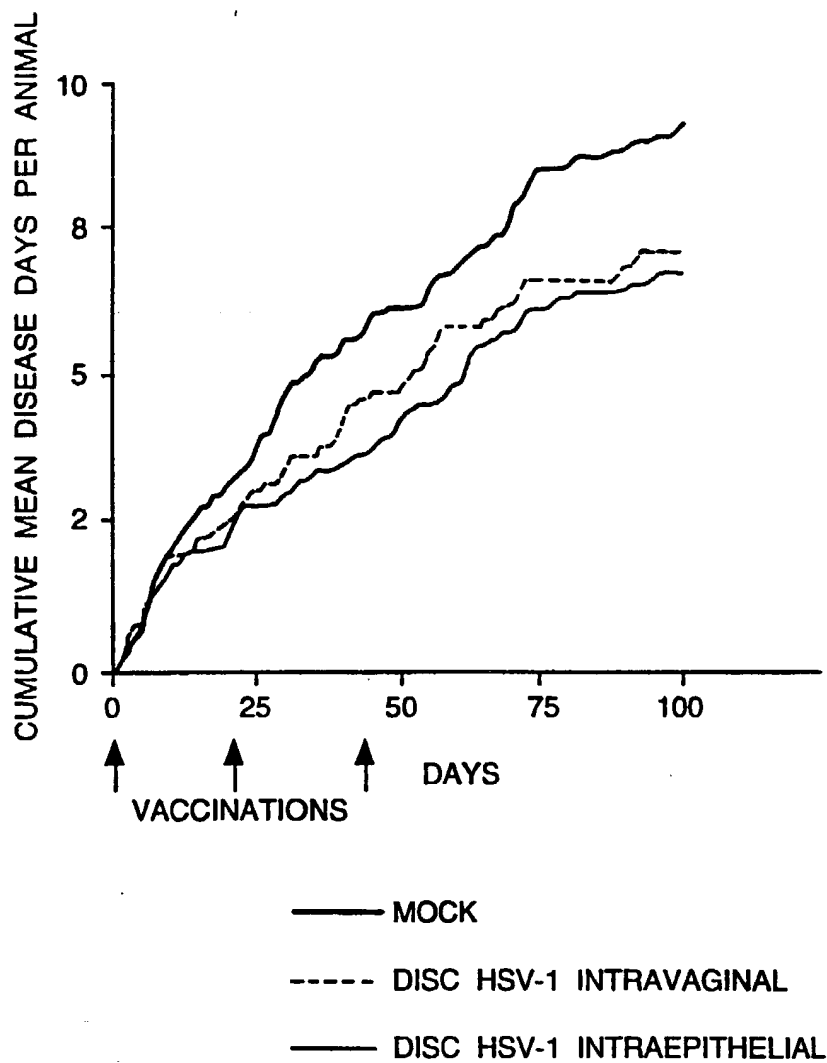


(c)



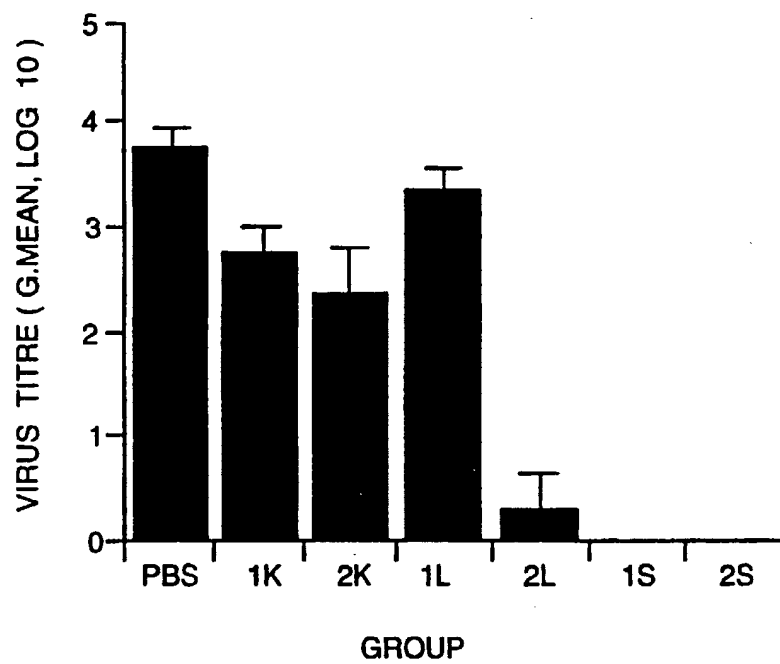
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Fig.13



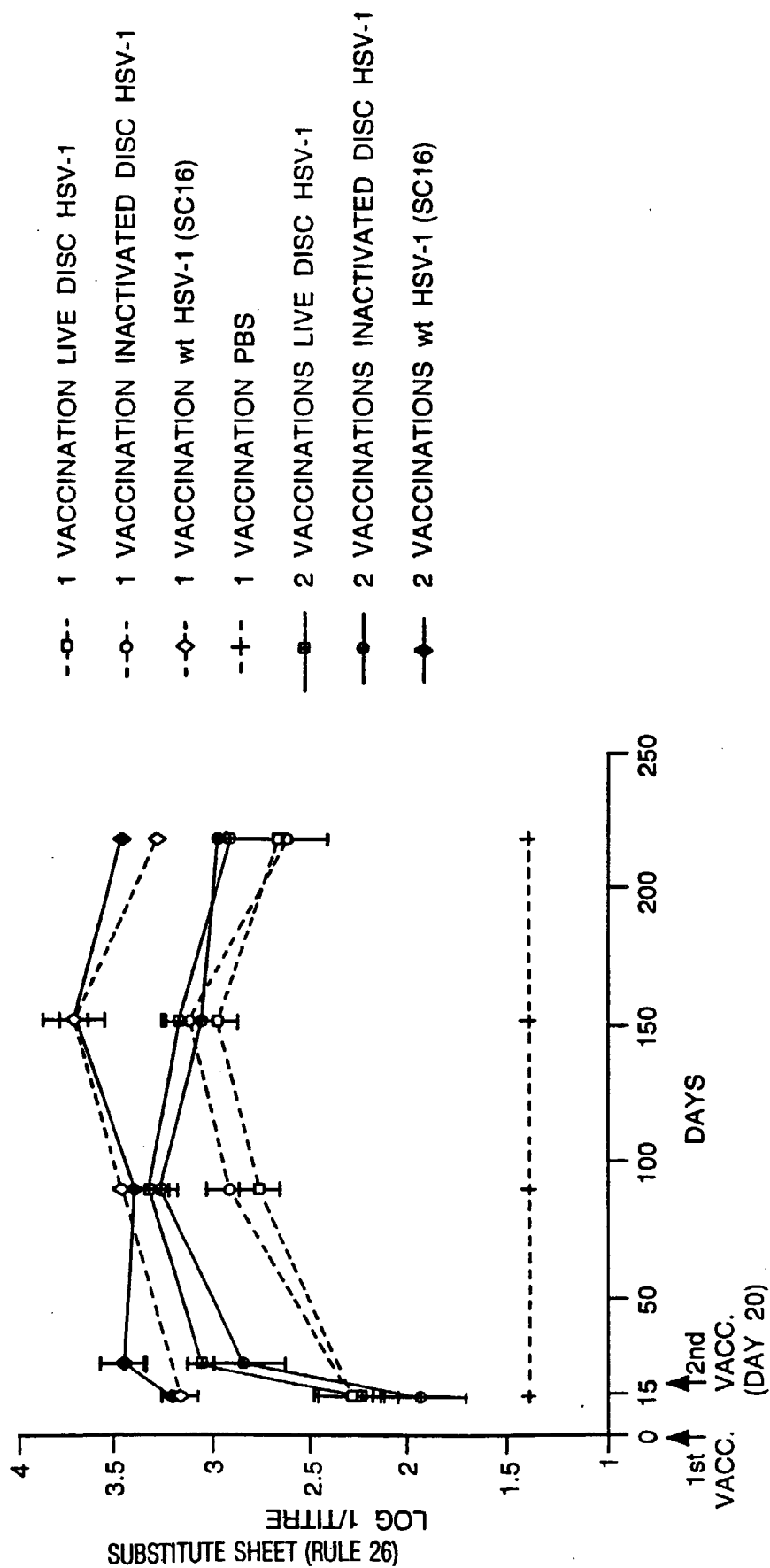
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Fig.14



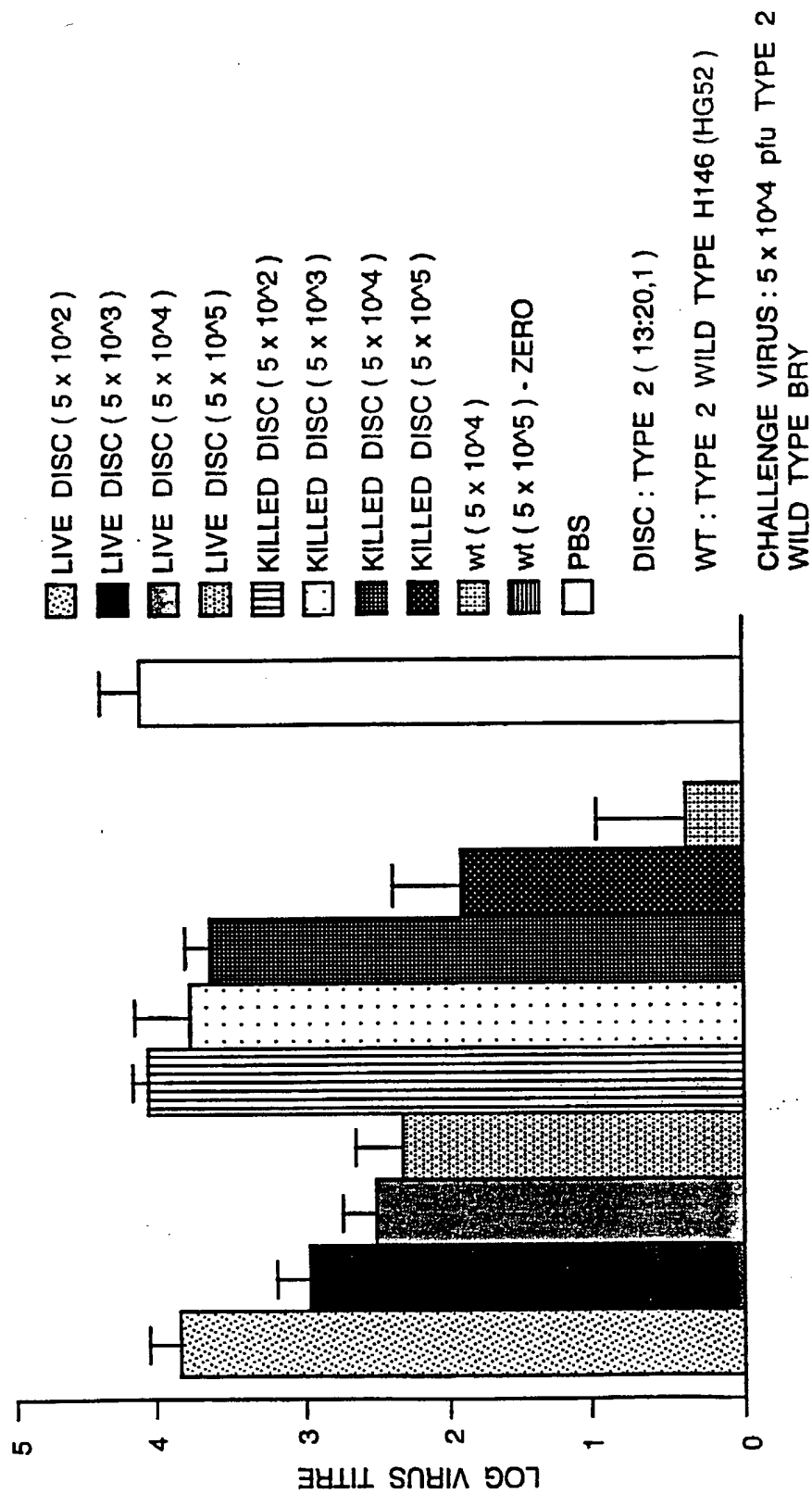
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Fig. 15



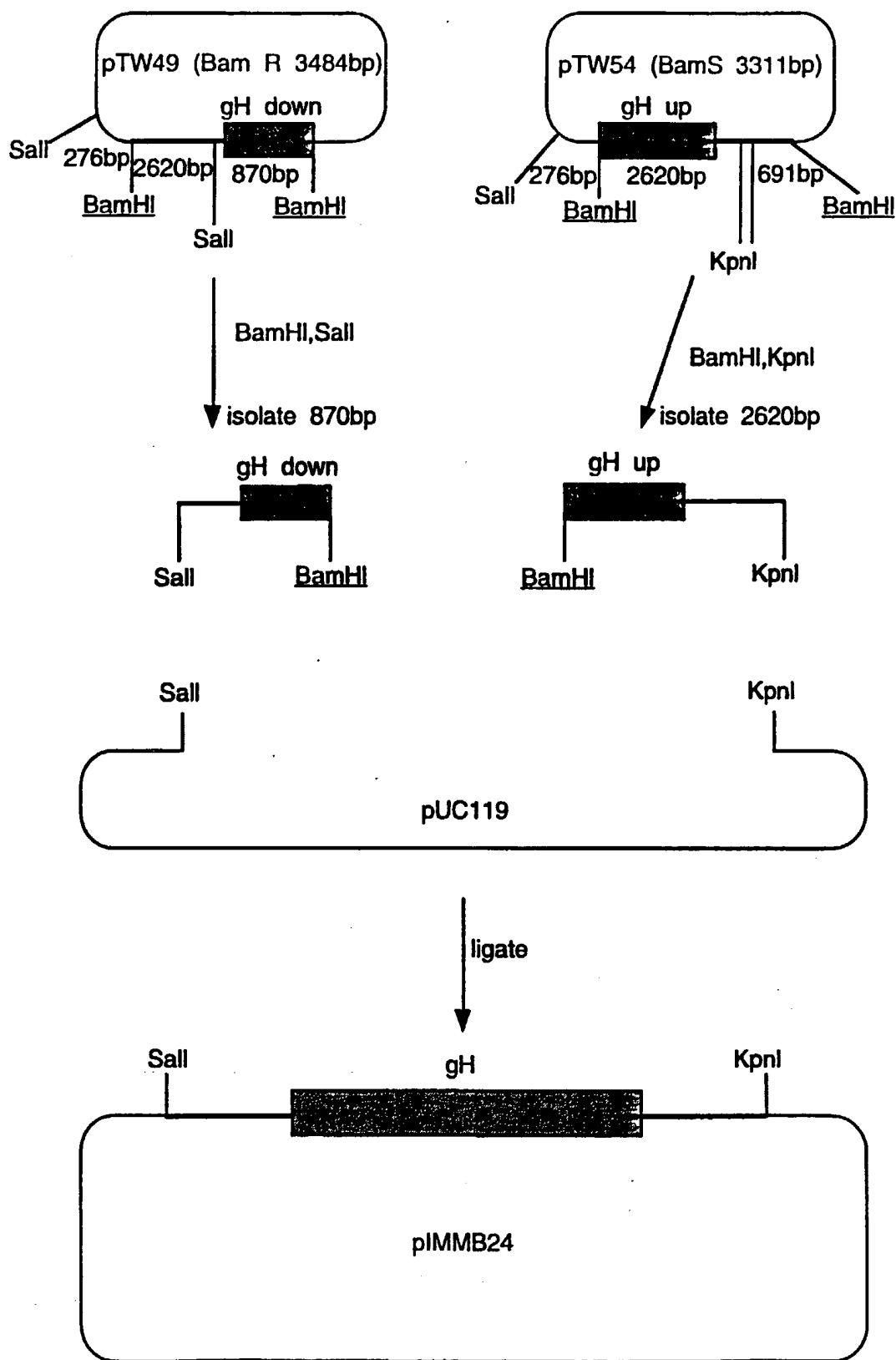
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Fig.16



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Fig.17.



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Fig.18

CTGCAGCGCGGCGGGAGGTGGCGGGAGGACTGGGGCCGGCTGACGGGGGTGCCCCGGGCG 60

ACCCCGCGCCCCGACCCCGAGGACGGCGGGGTCTCTGCCCCGCATCGAGGACACGCTG 120

TTTGCCCTGTTCCGCGTTCCTCGAGCTGCTGGCCCCCAACGGGGACTTGTACCACATTTTT 180

GCCTGGGTCTTGACGTCTTGCCGACCGCCTCCTTCCGATGCATCTATTTGTCCTGGAT 240

TACGATCAGTCGCCCCGTGGGTGTGAGACGCCCTGTTGCGCCTCACCGCCGGGATGATC 300

CCAACCCGCGTCACAACCGCGGGTCCATCGCCGAGATACGCGACCTGGCGCGCACGTTT 360

GCCCGCGAGGTGGGGGGAGTTTAGTTCAAACACGGAAGCCCGAACGGAAGGCCTCCCGGC 420

GATGACGGCAATAAAAGAACAGAATAAAAGGCATTGTTGTCGTGTGGTGTGTCCATAAGC 480

GCGGGGGTTCGGGGCCAGGGCTGGCACCCTATCAGCACCCACCGAAAAACGGAGCGGGC 540

CGATCCGTCCTTGTTTTCGGTCTGGTACTCCCTTTGTGCTTTTACCCTCACCCACCCCA 600

TCCTTTGGCCCGCGCTTACGGCAACAAGGGCCTCCGATAGCCTCCGAGGTGCGGACGCT 660

CTTTGGGCCGTGGGTACGGACACCCCCCATCTGCGGACTGGCAGCCGGGACGACGACCA 720

TGGGCCCCGCTCTGTGGGTGGTGATGGGGGTCTGGTGGNCGTTGCCGGGGGCCATGACA 780

G P G L W V V M G V L V V V A G G H D T

CGTACTGGACGGAGCAAATCGACCCGTGGTTTTTGCACGGTCTGGGGTTGGCCCGCACGT 840

Y W T E Q I D P W F L H G L G L A R T Y

ACTGGCGCGACACAAACACCGGGCGTCTGTGGTTGCCCAACACCCCGACGACCAGCGAC 900

W R D T N T G R L W L P N T P D D Q R P

CCCCAGCGCGGACGCTTGGCGCCCCGGGCAACTCAACCTGACTACGGCATCCGTGCCCA 960

P A R T L G A P G Q L N L T T A S V P M

TGCTTCGGTGGTACGCCGAGCGCTTTTGTTCGTGTGGTCAACACGGCCGAGTTTCCTC 1020

L R W Y A E R F C F V L V T T A E F P R

GGGACCCCGGGCAGCTGCTTTACATCCCAAAGACCTATCTGCTCGGCCGGCCTCGGAACG 1080

D P G Q L L Y I P K T Y L L G R P R N A

CGAGCCTGCCCGAGCTCCCCGAGGCGGGGGCCACGTCCCGTCCCCCGCGGAGGTGACCC 1140

S L P E L P E A G P T S R P P A E V T Q

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Fig.18(Cont 1)

AGCTCAAGGGACTGCTGCACAACCCCGGCGCCTCCGCGATGTTGCGGTCCCGGGCCTGGG 1200
L K G L L H N P G A S A M L R S R A W V

TAACATTTCGCGGCCGCGCCGACCGGAGGGGCTTACGTTNCCGCGGGGAGACGACGGG 1260
T F A A A P D R E G L T T P R G D D G A

CGACCGAGAGGCACCCGGACGGCCGACGCAACGCGNCCCGGGGCGCCCGCGGGGGCGC 1320
T E R H P D G R R N A A P G P P A G A P

CGAGGCATCCGACGACGAACCTGAGCATCGCGCATCTGCACAACGCGTCCGTGANCCTGC 1380
R H P T T N L S I A H L H N A S V V L L

TGGCCGCCAGGGGCTGCTACGGAATCCGGGTGCGTACGTGTACCTCTCCCGTCCGGCCT 1440
A A R G L L R T P G R Y V Y L S P S A S

CGACGTGGCCCGTGGGCGTCTGGACGACGGGCGGGTGGCGTTCGGGTGCGACGCCGCGC 1500
T W P V G V W T T G G L A F G C D A A L

TCGTGCGCGCGGATACGGGAAGGGCTTCATGGGGCTCGTGATATCGATGCGGGACAGCC 1560
V R A R Y G K G F M G L V I S M R D S P

CTCCGGCCGAGATCATAGTGGTGCCTGCGGACAAGACCCCTCGCTCGGGTCCGAAATCCGA 1620
P A E I I V V P A D K T L A R V G N P T

CCGACGAAAAACGCCCGCGTGCTCCCGCGCTCCGGCCGGCCCCAGGTATCGCGTCTTTG 1680
D E N A P R A P R A P A G P R Y R V F V

TCCTGGGGGCCCCGACGCCCGCGACAACGGCNTCGGCGCTGGACCCCTCGGCGGGTGG 1740
L G A P T P A D N G G G A G P P R R V A

CCGGCTACCCGAGGAGACGAACTACGCCCAGTATATGTGCGGGGCTATGCGGAGT 1800
G Y P E E S T N Y A Q Y M S R A Y A E F

TTTTGGGGGAGGACCCGGGCTCCGGCACGGACGACGCGCGTCCGTCCCTGTTCTGGCGCC 1860
L G E D P G S G T D D A R P S L F W R L

TCGCGGGGCTGCTCGCCTCGTCCGGGTTTTCGTTTCGTCAACGCGGGCCACGCCACGACG 1920
A G L L A S S G F A F V N A A H A H D A

CGATTCCGCTCTCCGACCTGCTGGGTTTTTTGGCCCACTCGCGCGTGTGGCCGGCCTGG 1980
I R L S D L L G F L A H S R V L A G L A

CCGCCCCGGGAGCAGCGGGCTGCGCGGCCGACTCGGTGTTCTGAACGTGTCCGTGTTGG 2040
A R G A A G C A A D S V F L N V S V L D

ACCCGGCGGGCCCGTCTGCGGCTGGAGGCGCGCTCGGGCATCTGGTGGCCGCGATCCTCG 2100
P A A R L R L E A R L G H L V A A I L E

AGCGAGAGCAGAGCCTGGCGGCGCACGCGCTGGGCTATCAGCTGGCGTTCGTGTTGGACA 2160
R E Q S L A A H A L G Y Q L A F V L D S

GCCCCGCGGCTATGGCGGGTTGGCCCCGAGCGGGCCCGCTGATCGACGCCCTTGTTA 2220
P A A Y G G L A P S A A R L I D A L V T

CCGCGCAGTTTCTCGGCGGCCGCGTAACGCCCCGATGGTCCGCGAGCGCTGTTTACG 2280
A Q F L G G R V T A P M V R R A L F Y A

CCACGGCCGTCTCCGGGCGCGTTCTGCGGGCGTGCCCTCGGCGGGCAGCGGGAAC 2340
T A V L R A P F L A G V P S A G Q R E R

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Fig.18(Cont 2)

GCCCCGGGGCCTCCTCATAACCACGGCCCTGTGTACGTCCGACGTGCGCGGGCGACCC 2400
P R G L L I T T A L C T S D V A A A T H

ACGCCGATCTCCGGGCGCGCTACGCAGGACCGACCACCAGAAAAACCTCTTCTGGCTCC 2460
A D L R A A L R R T D H Q K N L F W L P

CGGACCACTTTTCCCCATGCGCACGTTCCCTGCCGTTTCGATCTCGCCGAGGGCGGGTTCA 2520
D H F S P C A R S L P F D L A E G G F I

TCCTGGACGCGCTGGCCATGGCCACCCGATCCGACATCCCGGGCGGACGTCATGGCACAAC 2580
L D A L A M A T R S D I P A D V M A Q Q

AGACCCGCGGGCTGGCCTCCGCTCTCACGCNCTGGGCGACTCACAACGCCCTGATCCGCG 2640
T R G V A S A L T T W A T H N A L I R A

CCTTCGTCCCGGAGGCCACCCACCACTGTAGCGGCCGTCGCACAACGNGGAGCCCCGGA 2700
F V P E A T H Q C S G P S H N N E P R I

TCCTCGTGCCCATCACCACAACGCCAGCTACGTCGTCACCCACTACCCCCCTTGCCCCC 2760
L V P I T H N A S Y V V T H Y P P C P R

GCGGGATCGGATACAAGCTTACGGGCGTTGACGTCCGCGCCCGCTGTTTATCACCTATC 2820
G I G Y K L T G V D V R R P L F I T Y L

TCACCGCCACCTGCGAAGGGCACGCGGGGAGATTGAGCCGCGCGGCTGGTGCGCACCG 2880
T A T C E G H A R E I E P P R L V R T E

AAAACCGGCGGACCTCGGCCTCGTGGGGGCGTGTTCGCGCTACACCCGCGCGGGG 2940
N R R D L G L V G A V F L R Y T P A G E

AGGTCATGTCGGTGCTGCTGGTGGACACGGATGCCACCCAACAGCAGCTGGCCCAGGGGC 3000
V M S V L L V D T D A T Q Q Q L A Q G P

CGGTGGCGGGCACCCGAACGTGTTTCCAGCGACGTGCCGTCGTCGCGCTGTTGTTGT 3060
V A G T P N V F S S D V P S V A L L L F

TCCCCAACGGAACGTGATTTCATCTGCTGGCCTTTGACACGCTGCCCATCGCCACCATCG 3120
P N G T V I H L L A F D T L P I A T I A

CCCCGGGTTTCTGGCGCGTCCGCGCTGGGGGTCGTTATGATTACCGCGGCCCTGGCGG 3180
P G F L A A S A L G V V M I T A A L A G

GCATCCTCAGGGTGGTCCGAACGTGCGTCCCATTTTGTGGAGACGCGAATAAACGGGTG 3240
I L R V V R T C V P F L W R R E *

TGTGGACGCAGCGCGTCCAGCCCAACCCAACCGACTCCCTCCGTGTCGCGGTCTGTTT 3300

GTTATTGTGTCCGCGTGGCTCCGCTACCGCCTCTGTTCCCTTTCCCTTCTCCATTCTGT 3360

TTCTTTCTTTCCCCCCCCCATAGTCCCCCGTATAGGCATACAACGGCATCCGTGGGT 3420

End of HSV2 UL21

TAGAAAACGACTGCACTTTATTGGGATATCTACACAGACTGGCCGTGCTGGGCGCGAGC 3480
* V S Q G H

CAGGCAAACGGTAAGCAGCGCGTCCAGGTACCGGCGGTTGCGGTGCGGCCAGCCGCCCC 3540

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Fig.18(Cont 3)

| | |
|--|------|
| CGCCGGCCCGCGGTCAAACGCGGACATCCGGTCGACGTCCCCACGGTCAGGACCAGGGA | 3600 |
| CGTCACGCCCCTCAGGCGCNCGGTATGCGTGGCCGCGGCCAGGCGTCCGTGGCCGGCGTA | 3660 |
| CAACACGCCCAGGAACGCGCCGAGGTACATGACGTGCTCGGGCGAGACGGACCCCCCGG | 3720 |
| GGTCAGGCGTTGCGAGTCCACAAAGCGCAGCAGGGCGGCGCTGTCGGCCCGCGACGTCCG | 3780 |
| TCCCCACCGGCACGTCCTTGGGCGGGAGGAGGTCGAACATGAGGAGCTGCTCGCGA | 3840 |

Fig. 19. (Cont 2).

| | | |
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| 11850 | GCTTTCTGTAACGCCGCCACCAAGCGCGGCTCTCCGACTGCTAGGCTTTTGGCCCACTCGCGCGGCTTGGCGGTTGGCCCGCCGCG | 1949 |
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| 11858 | | |
| 11859 | | |
| 11860 | CGCTTCGTCACGCGGCCACGCCACGACGCGATTTCGCTCTCCGACTGCTGSGTTTGGCCCACTCGCGCTGCTGGCGGCTGGCCCGCGCGG | 1989 |
| 11861 | | |
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| 11863 | | |
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| 11890 | GAGCAGCGGGTGGCGGCCGACTCGGTGTTCTGTAACGTTCGCTGTTGGACCGCGGCCCGTCTGCGGCTGGAGCGCGCTCGGGCATCTGTTGGC | 2089 |
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| 11904 | | |
| 11905 | GGGCGCGGGGTGTCCGCGGATTCTGTGTTTTTAAATGTGTCAGTCTTGATCCACAGCGCCGCCCTGCAGCTAGAGGCTCGGCTCCAGCACCTGTGTGGC | 2049 |
| 11906 | | |
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| 11924 | | |
| 11925 | CGAGATTCTGAGCGCGGAACAGAGCTTGGCAATTACAGCGCTGGGCTATCAGCTGSCCTTCGTGCTGGATAGCCCCCTCGGCTACGACGAGTGGCGSCCC | 2149 |
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| 11927 | | |
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| 11950 | CGCGATCCTCAGCGAGAGCAGGCTGGCGGCGCACGCGCTGGGCTATCAGCTGGGTTCTGTTGGACAGCCCCCGGCGCTATGGCGGTTGGCCCCG | 2189 |
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| 12000 | | |

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Fig.20.

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1  MGNGLWFGVVIILGVANGQVHDWTEQTDPFWLDGLGMDRMYWRDNTNGRLWLPNTDPDPQK 60
  :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: ::
1  MGPGGLW VVMGVLVAGGHDYWTEQIDPFWLHGLGLARTYWRDNTNGRLWLPNTDDQOR 59

61  PPRGFLAPPDELNLTASLPLLRWYEERFCFVLVTTAEFPRDPGQLLYIPKTYLLGRFPN 120
  :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: ::
60  PPARTLGAPGQLNLTASVPMRLWYAERFCFVLVTTAEFPRDPGQLLYIPKTYLLGRFRN 119

121  ASLPAPTVEPTAQPPPSVAPLKGLLHNPAAASVLLRSRAWVTFSAPVDPPEALTFFPRGDNV 180
  :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: ::
120  ASLPELPEAGPTSRPPAEVTQLKGLLENPGASAMLSRAWVTFAAAPDREGLT PRGDDG 178

181  ATASHPSGPRDTPPPRPPVGARRHPTTELDITHLHNASTTWLATRGLLRSPGRYVYFSPS 240
  :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: ::
179  ATERHPDGRRNAPG PPAGAPRHPTTNLSIAHLHNAS VLLAARGLLRTPGRYVYLSPS 235

241  ASTWPGVIWTTGELVLGCDAAALVRARYGREFMGLVISMHDSPPVEVMVVPAGQTLDRVGD 300
  :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: ::
236  ASTWPGVWTTGGLAFGCDAAALVRARYGKGFMGLVISMRDSPPAEIIVVPADKTLARVGN 295

301  PADENPPGALPGPPGGPRYRVFVLGSLTRADNGSALDALRRVGGYPPEGTNYAQFLSRAY 360
  :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: ::
296  PTDENAPRA PRAPAGPRYRVFVLGAPTPADNGGA GPPRRVAGYPEESTNYAQYMSRAY 353

361  AEFFSGDAG AEQGPRPPLFWRLTGGLATSGFAFVNAAHANGAVCLSDLLGFLAHSRALA 419
  :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: ::
354  AEFLGEDPGSGTDDARPSLFWRLAGLLASSGFAFVNAAHAHDAIRLSDLLGFLAHSRVLA 413

420  GLAARGAAGCAADSVFFNVSVLDPTARLQLEARLQHLVAEILEREQSLAHLALGYQLAFV 479
  :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: ::
414  GLAARGAAGCAADSVFLNVSVLDPAARLRLEARLGHVAAILEREQSLAAHALGYQLAFV 473

480  LDSPSAYDAVAPSAHLIDALY AEFLGGRVLTTPVVHRALFYASAVLRQPFLLAGVPSAV 538
  :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: ::
474  LDSPAAYGGLAPSAARLIDALVTAQFLGGRV TAPMVRRALFYATAVLRAPFLAGVPSAG 532

539  QRRARRRSLLIASALCTSDVAAATNADLRRTALARADHQKILFWLPDHFSPCAASLRFDLD 598
  :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: ::
533  QRRERP RGLLITTALCTSDVAAATHADLRALRRTDHQKNLFWLPDHFSPCARSLPFDLA 591

599  ESVFILDALAQAATRETPVEVLAQOQTHGLASTLTRWAHYNALIRAFVPEASHRCGGQSAN 658
  :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: ::
592  EGGFILDALAMATRS DIPADVMAQOTRGVASALT WATHNALIRAFVPEATHQCSGSPSHN 650

659  VEPRILVPITHNASYVVTH SPLPRGIGYKLTGVDVRRPLFLTYLTATCEGSTRDIESKR 717
  :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: ::
651  EPRILVPITHNASYVVTHYPPCPRGIGYKLTGVDVRRPLFITYLTATCEGHAREIEPPR 709

718  LVRTONQORDLGLVGAVFMRYTPAGEVMSVLLVDTDNTQQQIAAGPTEGAPSVFSSDVPST 777
  :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: ::
710  LVRTENRRDLGLVGAVFLRYTPAGEVMSVLLVDTDATQQQLAQGPVAGTPNVFSSDVPST 769

778  ALLLFPNGTVIHLLAFDTQPVAAIAPGFLAASALGVVMITAALAGILKVLRTSVFFWRR 837
  :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: ::
770  ALLLFPNGTVIHLLAFDTLPATIAPGFLAASALGVVMITAALAGILRVVRTCVPFLWRR 829

838 E 838
  :
830 E 830

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Fig.21.

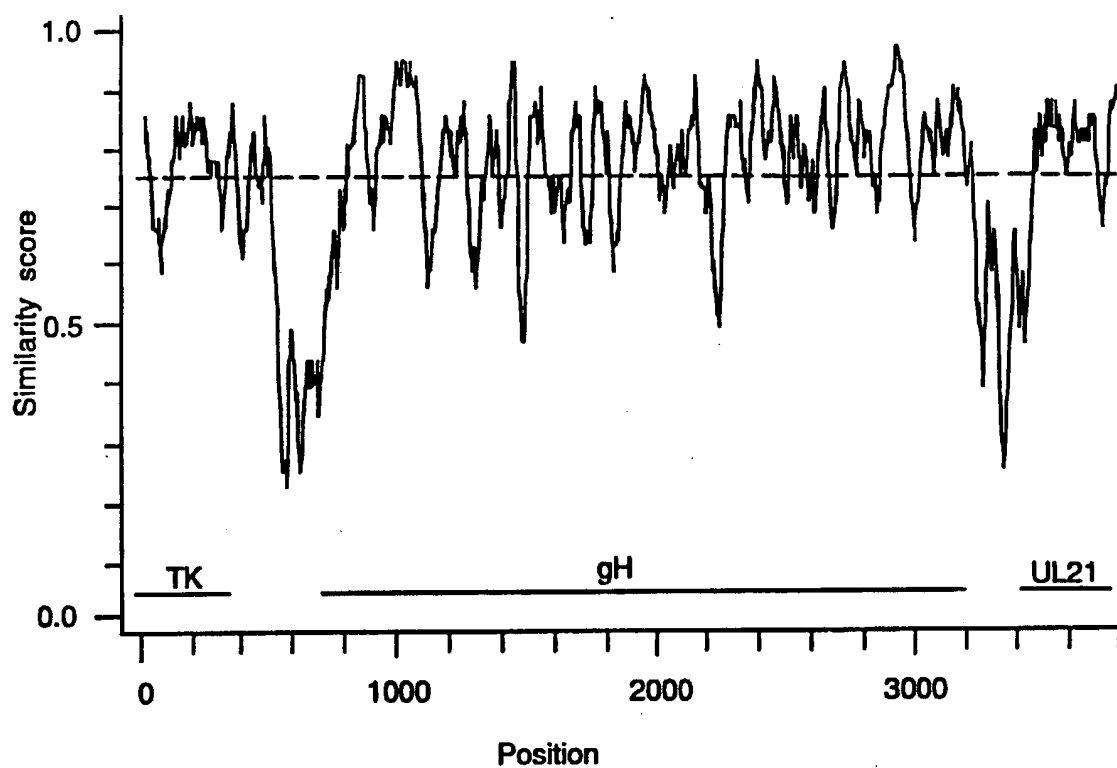
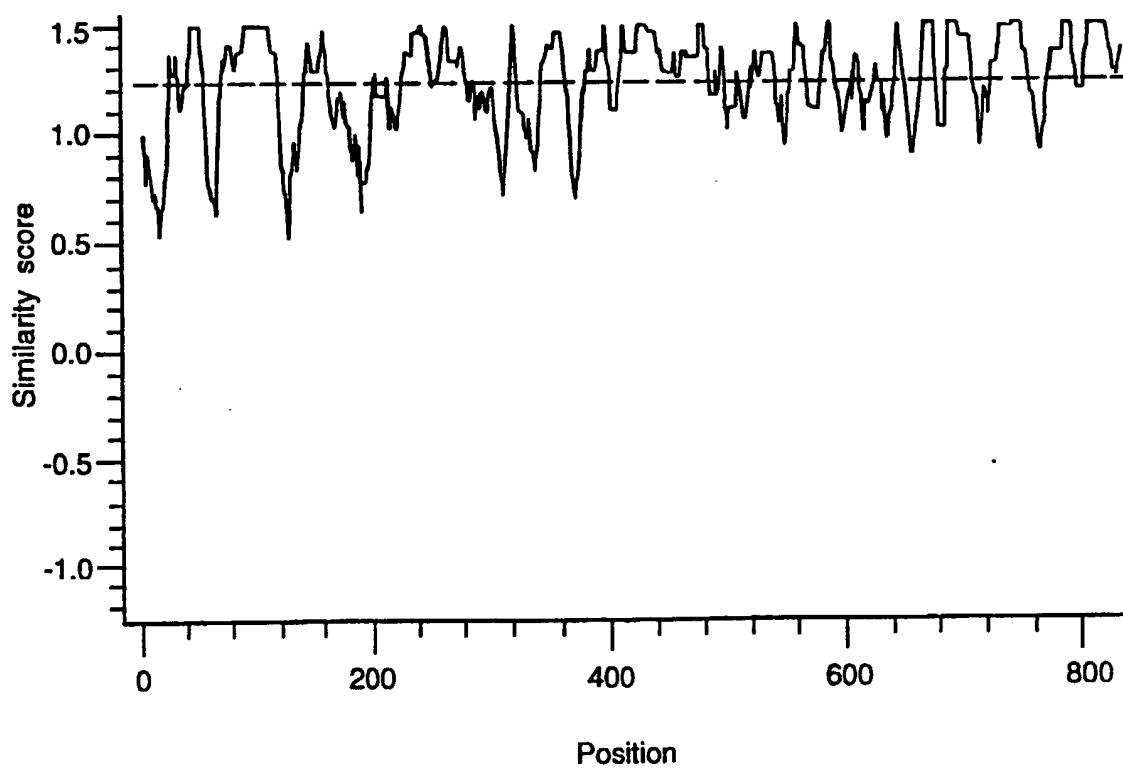
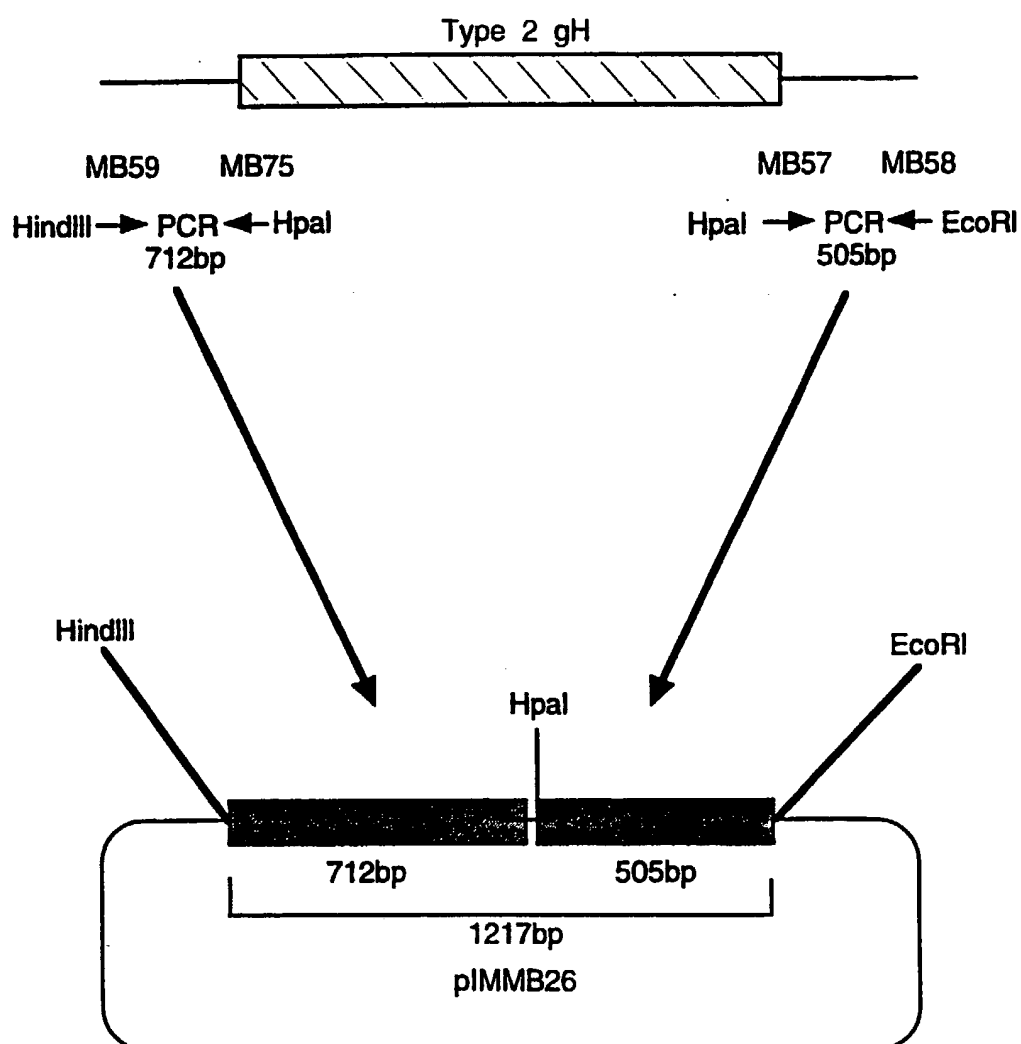


Fig.22.



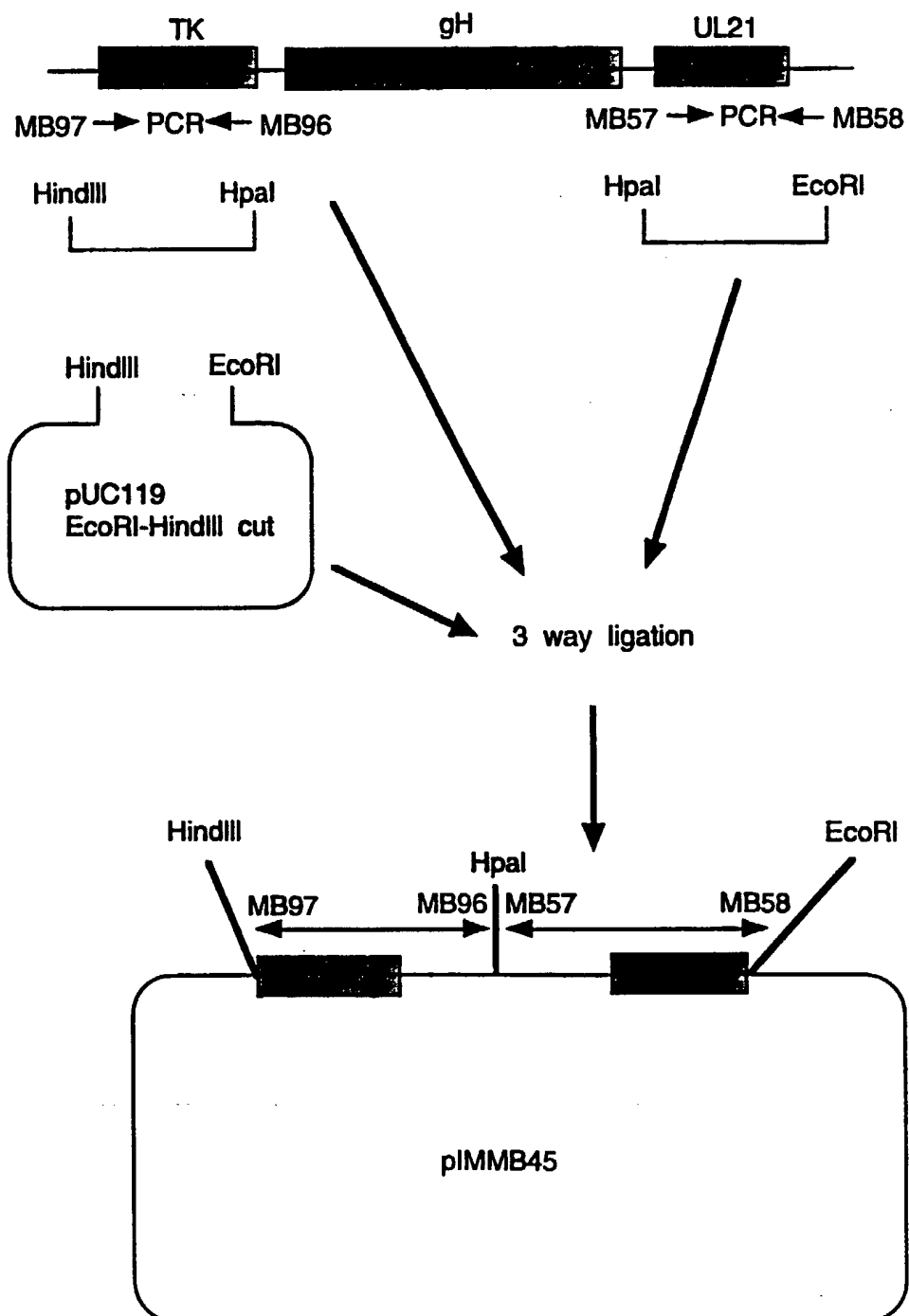
28/33

Fig.23.



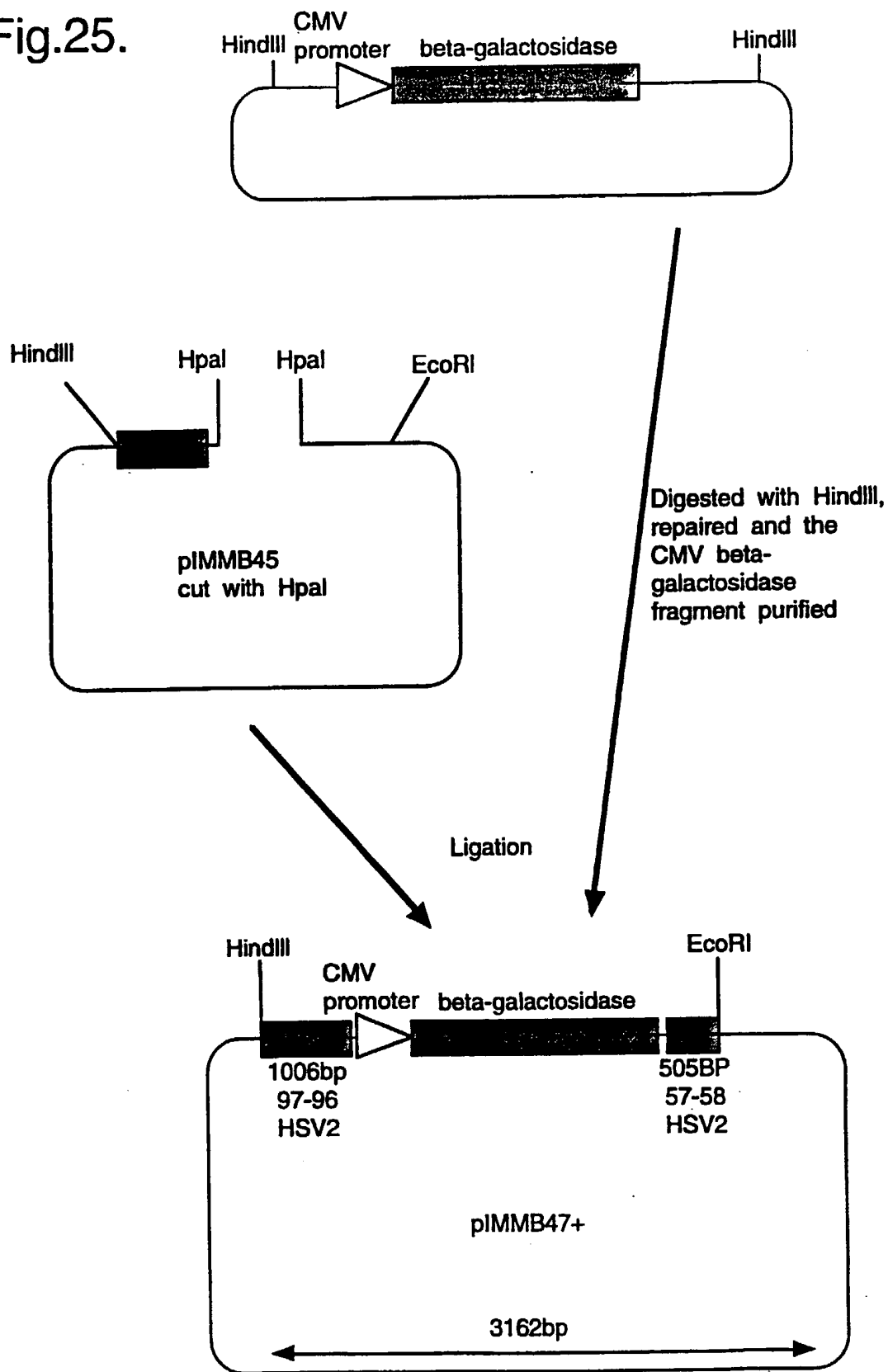
29/33

Fig.24.



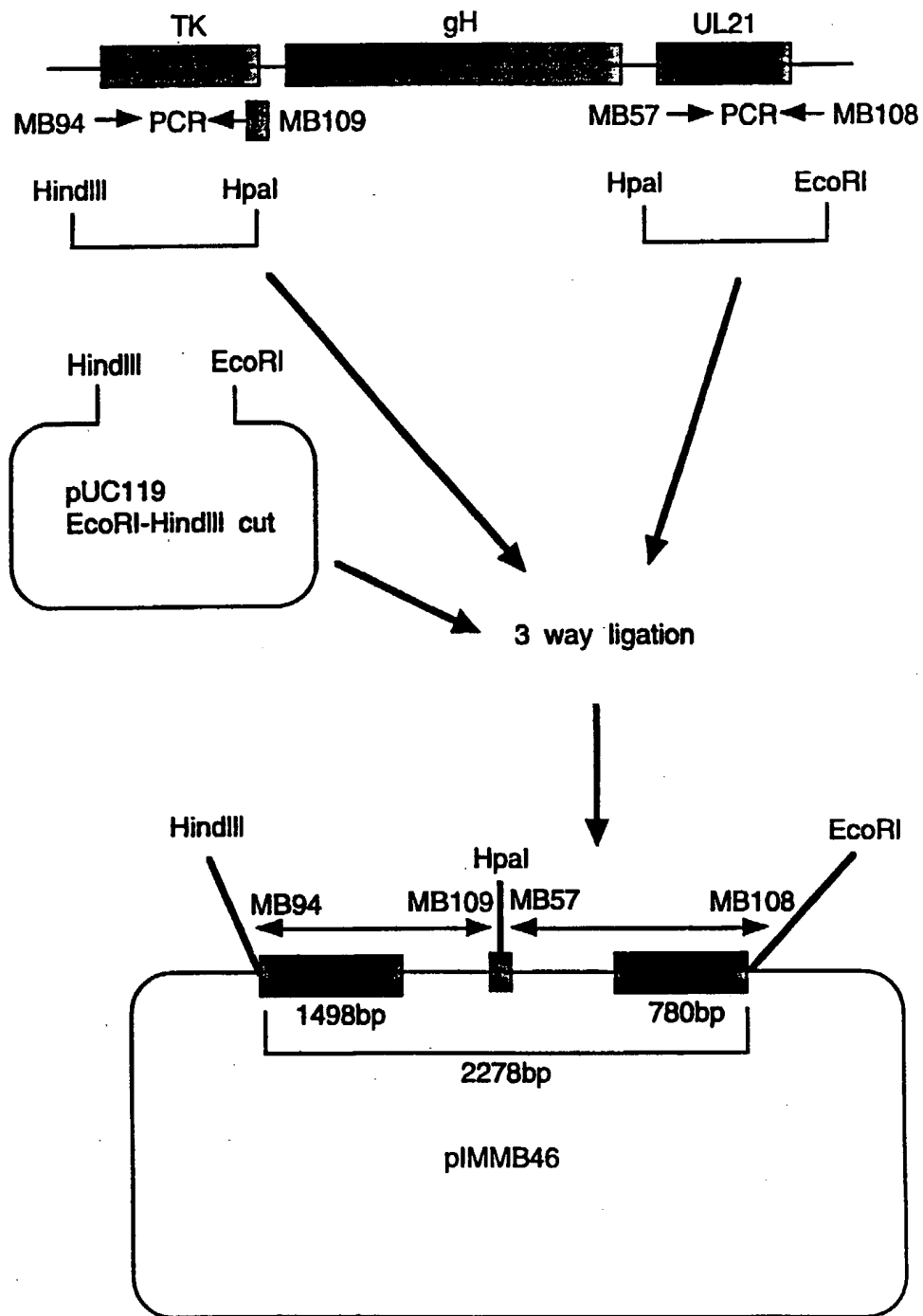
30/33

Fig.25.



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Fig.26.



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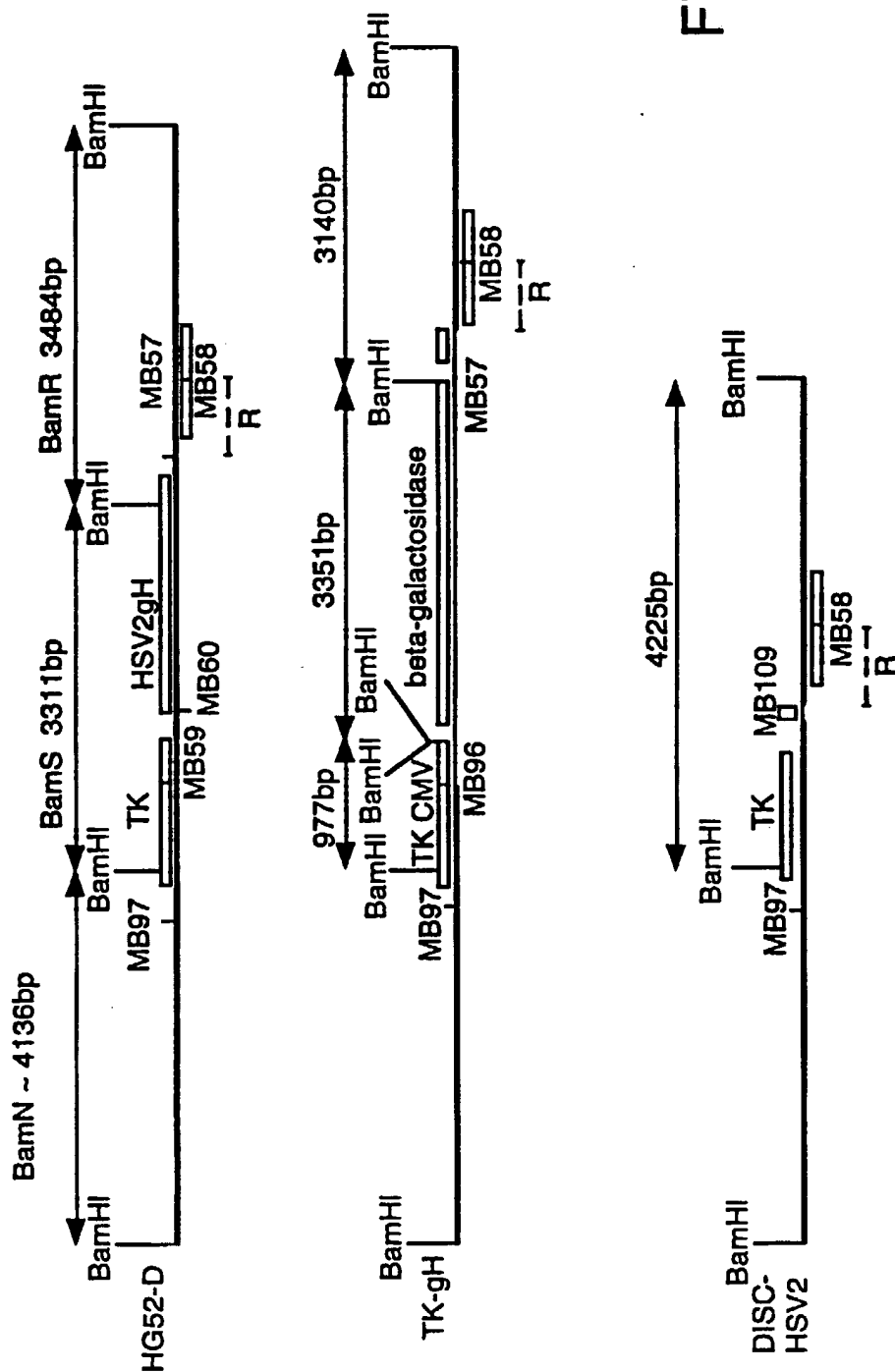
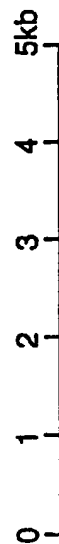
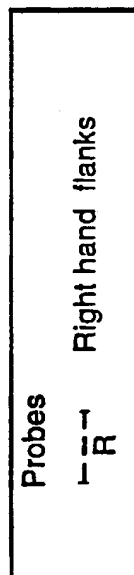
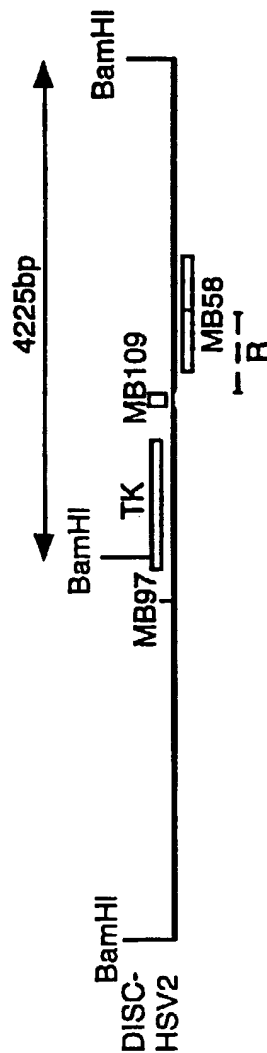
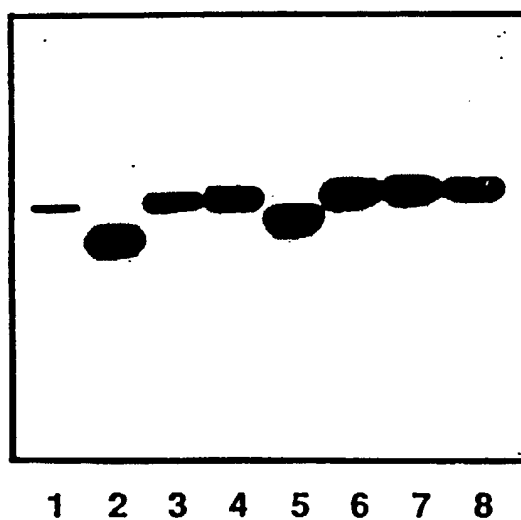


Fig.27.



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Fig.28.



INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 94/00572

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C12N15/86 C12N7/04 A61K39/245 C12N15/38

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|--|---|
| X | JOURNAL OF CELLULAR BIOCHEMISTRY vol. SUPPL, no. 17D, 13 March 1993 page 84 MCLEAN, L. ET AL. 'Protection against HSV-2-induced disease by vaccination with a GH-deleted HSV-1 virus' see abstract N 422 | 1-4, 6, 7, 9, 11-14, 17-19, 22-26, 29-33, 35, 37 |
| O, X | & Keystone symposium on molecular immunology of virus infections TAOS, USA, MARCH 17-24 1993 --- -/- | 1-4, 6, 7, 9, 11-14, 17-19, 22-26, 29-33, 35, 37 |

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

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 "E" earlier document but published on or after the international filing date
 "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
 "O" document referring to an oral disclosure, use, exhibition or other means
 "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

18 July 1994

Date of mailing of the international search report

23. Ud. 94

Name and mailing address of the ISA

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Fax: (+31-70) 340-3016

Authorized officer

Chambonnet, F

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 94/00572

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|----------|---|---|
| X | WO,A,92 05263 (IMMUNOLOGY LTD) 2 April 1992 cited in the application | 1-4,6,7, 11-14, 16, 22-26, 28,30-34 |
| Y | see the whole document | 5,8-10, 15, 17-21, 27,29, 36,37 |
| X,P | ----- JOURNAL OF VIROLOGY vol. 68, no. 2, February 1994 pages 927 - 932 FARRELL, H.E. ET AL. 'Vaccine potential of Herpes Simplex Virus type 1 mutant with an essential glycoprotein deleted' | 1-4,6,7, 11-14, 16, 22-26, 30-34 |
| P,Y | see the whole document | 5,8-10, 15, 17-21, 27-29, 36,37 |
| Y | ----- FIELDS, B.N. & KNIPE, D.M. 'VIROLOGY' 1991, RAVEN PRESS, NEW YORK, USA see page 1847 - page 1849 | 5,8-10, 15,28 |
| P,X | WO,A,94 01573 (AKZO N.V.) 20 January 1994 see the whole document | 1-4,6,8, 11,12, 22-24, 30-32 |
| P,X | WO,A,94 03595 (AKZO N.V.) 17 February 1994 see the whole document ----- | 1-4,6,8, 11,12, 22-24, 30-33 |

INTERNATIONAL SEARCH REPORT

Inte. ional application No.

PCT/GB94/00572

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 30-37 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 94/00572

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---------------------|----------------------------|---------------------|
| WO-A-9205263 | 02-04-92 | AU-A- 8648991 | 15-04-92 |
| | | CA-A- 2091678 | 26-03-92 |
| | | EP-A- 0550553 | 14-07-93 |
| | | GB-A, B 2263480 | 28-07-93 |
| | | JP-T- 6504194 | 19-05-94 |
| WO-A-9401573 | 20-01-94 | AU-B- 4589893 | 31-01-94 |
| WO-A-9403595 | 17-02-94 | AU-B- 4560693 | 03-03-94 |

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| Total (1) | 97 | 97 | 0 | - |